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13. ABSTRACT (Maximum 200 Words) In the period covered by this application, we have established that the PC-346C ^{RFP} can be used as a suitable model for androgen dependent localized prostate cancer. We have shown that orthotopic tumors derived from PC-346C ^{RFP} cells (inoculated in growth factor replete Matrigel into the ventral prostate of nude mice) are responsive to Asodex, and undergo tumor regression as a consequence of an increase in apoptosis and a decrease in proliferation rate. LNCap cells selected on the basis of their invasive potential in vitro however do not respond to Casodex and do not metastasize to other organs. We attribute this to the presence of a mutated Androgen receptor in the LNCaP cells which responds to the adrenal steroid dehydroepiandrosterone (DHEA), overriding the effects of Casodex, and to the high levels of growth factors. When inoculated in growth factor depleted Matrigel PC-346C ^{RFP} cells form primary tumors, and after extended treatment with Casodex, show evidence of metastatic progression after treatment with Casodex, although the data has not yet reached statistical relevance. Micro-array and macro-array techniques are being used to characterize the expression of genes induced by Casodex in this tumor to identify the genes that may underlie tumor progression.				
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Introduction

In 2000, an estimated 180,4000 men were diagnosed with prostate cancer in the United States, and 31,400 succumbed to the disease (American Cancer Society Facts and Figures, 2000). Due to the increase in public awareness and the greatly increased use of Prostate Specific Antigen (PSA) for screening, prostate cancer now is the second most commonly diagnosed male cancer in many western countries after lung cancer. The major risk factors for prostate cancer include age and race, and the consumption of a high fat diet. The main cause of death from prostate cancer is the invasion and metastasis of prostate cancer to the bone, liver and brain. However, for many men (approximately 100,000 of those diagnosed each year) the disease will remain localized and slow growing. Extensive PSA screening programs have lead to the increased identification of early stage (A1 and A2) tumors in younger men. Approximately 70% of these tumors are indolent and will not need treatment during the patients life time (Choo et al., 2002). Unfortunately at present there is no way to distinguish between aggressive, clinically significant tumors that need to be treated and indolent tumors. As a result, many patients are treated more aggressively than is necessary.

There are four major strategies for treatment of localized, early stage prostate cancer: radical prostatectomy, radiation therapy (either external beam, three dimensional conformal therapy or brachytherapy), hormone therapy (usually with Casodex or flutamide with or without an LH-RH agonist such as Zoladex) and watchful waiting (waiting for the PSA levels to rise before deciding on a course of treatment). The combined five year survival for these interventions is approximately 75 %, however the majority of recurrent tumors develop resistance to further therapeutic intervention. The recent Bicalutamide 150mg (Casodex) Early Prostate Cancer (EPC) Program was established to examine whether adding 150mg/day Casodex immediately to standard care (watchful waiting, radical prostatectomy or radiotherapy) reduces the risk of disease progression and improves survival when compared to standard care alone. Analysis of the data from the EPC Program, which enrolled 8,113 patients with localized and locally advanced prostate, shows that Casodex cuts the risk of disease progression by almost half in patients with localized or locally advanced prostate cancer, and also demonstrates that the time to prostate-specific antigen (PSA) doubling was significantly delayed in patients receiving Casodex and standard care compared with standard treatment alone (Wirth et al., 2001; Wirth, 2001; Iversen et al., 2002). As a result, there is a very significant increase in the number of patients being treated with Casodex, either alone or immediately after surgery or radiation. Furthermore, neoadjuvant therapy with Casodex to debulk organ-confined prostate tumors (particularly stage B1) and to improve positive margins is now widely used prior to surgery and radiation therapy (Padula et al., 2002), and many 'at risk' men (defined as men with two first degree relatives with prostate cancer) are now considering chemoprevention in the form of Casodex (Trump et al., 2001; Schellhammer, 2002).

The aim of the studies funded by this award is to examine the effects of Casodex and other anti-androgens on the induction of apoptosis in androgen dependent PC-346C and LNCaP human prostate cancer cells, and to understand the molecular basis of tumor progression. These cell lines are being used as a model of early, organ confined androgen dependent prostate cancer. One of the major unresolved issues in the development of prostate cancer is the mechanism underlying the progression from hormone dependent to hormone refractory prostate cancer after treatment with anti-androgens. Since there are an increasing number of men being treated with Casodex mono-therapy for localized prostate cancer, as a result of the initial success of the 150mg (Casodex) Early Prostate Cancer (EPC) Program, it is important to fully evaluate the biological effects of Casodex to ensure that it does not induce adverse effects.

Body

The experimental aims for this operating grant are:

Task 1: Analysis of PC-346C cells (months 1-8) to determine the effects of Casodex on apoptosis and cell cycle, determine whether Casodex or flutamide can induce an invasive phenotype, to monitor changes in gene expression using RT-PCR and to clonally expand the invasive cells for further study.

Task 2: Determine the metastatic capability of the invasive cell lines produced above, both in vitro and in vivo using the orthotopic xenograft model system. (months 8-20).

Task 3: Examine the induction of the invasive phenotype in LNCaP^{GFP} and PC-346^{RFP} cells, and to characterize the changes in gene expression induced by Casodex. (months 8-20). Initiated, in progress

Task 4: Identify differentially expressed genes using microarray technology (months 3 -36)

With respect to **Task 1**, the experiments have been completed on schedule, and we have written two manuscripts (Zhan et al., 2003 and Lee et al., 2003) that have been published since the last annual report. (See appendix 1 and 2). Briefly we have shown that Casodex induces cell death via an intracellular signaling pathway that is distinctly different from the mechanism of action of TNF α . Treatment of androgen sensitive, non-metastatic LNCaP human prostate cancer cells with 0-100 μ M Casodex or 0-10 ng/mL TNF α induces cell death in 20-60% of the cells by 48 h in a dose dependent manner. However, Casodex does not induce classical DNA fragmentation to oligonucleosomes typically induced by TNF α , but rather induces cleavage to form intermediate 60 kb DNA fragments. RT-PCR based analysis demonstrates that in LNCaP cells Casodex coordinately alters the expression of steady state level of mRNAs of several matrix metallo-proteases and their cognate inhibitors (most notably MMP-2 and TIMP-1). Zymography and reverse zymography confirm that the ratio of metallo-protease(s) to inhibitor(s) is altered in favor of activation of the proteases. In cells treated with TNF α , this is accompanied by the loss of mitochondrial membrane potential ($\Delta\Psi$ m) and cell adhesion. In contrast, cells treated with Casodex display loss of cell adhesion, but sustained mitochondrial dehydrogenase activity. Over-expression of Bcl-2 in LNCaP cells attenuates the induction of cell death by TNF α but not Casodex, suggesting that mitochondria depolarization is not required for the induction of cell death by Casodex. While TNF α induces release of cytochrome c in LNCaP cell is associated with the translocation and cleavage of Bax, Casodex-induced cytochrome c release involves both Bax-dependent and -independent pathways, suggesting that Casodex induces cell death by acting on components downstream of decline of $\Delta\Psi$ m and upstream of cytochrome c release. Furthermore, while induction of both caspase-3 and caspase-8 activities are observed in TNF- α and Casodex-treated cells, a novel cleavage product of pro-caspase-8 is seen in Casodex-treated cells. Taken together, these data support the hypothesis that Casodex induces cell death in an indirect and incomplete fashion that is independent of changes in $\Delta\Psi$ m and Bcl-2 actions and results in an extended lag phase of cell survival that may promote the induction of an invasive phenotype after treatment. Thus, different drugs may induce cell death in the same cell line through different mechanisms that involve many or all of the same components of the apoptotic machinery, but with substantially different time course and efficiency. In a small percentage of the treated LNCaP cells, the activation of the ECM-proteases by Casodex also induces an invasive phenotype. The acquisition of an invasive phenotype is not seen when LNCaP cells are treated with TNF α , and is not seen when the LNCaP cells are treated with both compounds simultaneously, suggesting that the phenomenon may be specific to particular classes of compounds. These experiments offer a mechanistic explanation for the failure of most anti-androgen therapies in prostate cancer and the emergence

of hormone refractory tumors that have high propensity for metastasis, and raises questions about the use of Casodex and other anti-androgens for neo-adjuvant therapy or as chemopreventive agents (Zhan et al 2003; Lee et al., 2003)

With regard to the experiments outlined in **Task 2**, we have found that the LNCaP sublines, I-1 and I-33, when grown as xenografts in nude mice grow slowly as well encapsulated primary tumors that metastasized infrequently to other organs (4/50 animals for each subline). We first isolated these cell lines from the invasive LNCaP population that transversed the 8 μ membranes in the Boyden chamber assay. *In vitro* these cell lines grow rapidly and are consistently very invasive, however in the xenograft model these cells form relatively slow growing tumors and do not appear to be particularly aggressive. There are possible reasons for this low rate of metastasis:

First, in contrast to their invasive phenotype *in vitro*, these cells may not be intrinsically metastatic *in vivo*. If the invasive phenotype is not merely an *in vitro* artifact, this would suggest that the acquisition of an invasive phenotype is reversible and is dependent on either intrinsic or extrinsic signaling to maintain the invasive phenotype. This signaling is presumably not active in the xenografts, or is overridden by other (extrinsic) factors. These factors may include the growth factors present in the Matrigel used during the inoculation of the cells into the mammary fat pad.

Secondly, it is well established that the mutation of the androgen receptor present in the LNCaP cells renders the receptor promiscuous, and results in the agonistic activation of the receptor by the adrenal steroid dehydroepiandrosterone (DHEA), which is produced in milligram quantities by the rodent adrenal gland. This agonistic activation of the AR may block or severely blunt the signaling by Casodex that leads to the initiation of apoptosis and metastasis. These two issues appear to confound the successful completion of this task using the experimental approaches initially proposed. However to circumvent these problems we have performed an additional experiment that deviates slightly from the original SOW. The description of this experiment, which utilizes the PC-346C^{RFP} cells has been included under **Task 3b**, and essentially utilizes this new cell line to test the hypothesis that treatment with Casodex induces metastatic progression.

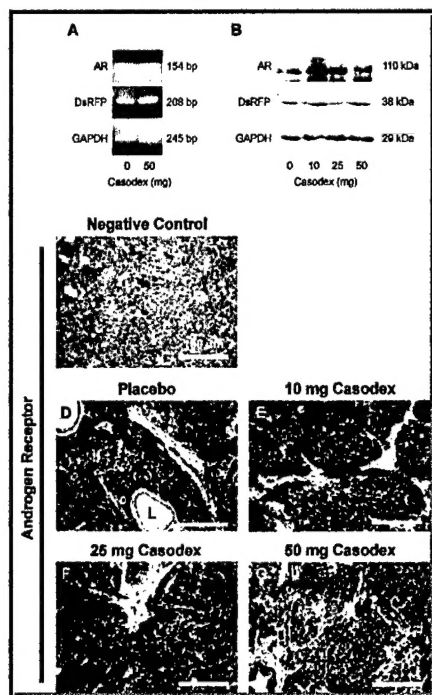


Figure1: Expression of AR in PC-346C^{RFP} tumors treated with Casodex. Panel A: RT-PCR of AR mRNA compared to GAPDH and RFP mRNAs. Panel B: Western analysis of AR expression compared to RFP and GAPDH. Panel C: Immunohistochemistry of AR in tumor cells treated with increasing doses of Casodex. androgen

Task 3a: We have created stable PC-346C cell lines expressing red fluorescent protein RFP (PC-346C^{RFP}) by limiting dilution after transfection with a RFP expression vector and selection with G418. These cell lines undergo cell cycle arrest and apoptosis in a time and dose dependent manner in response to Casodex that is essentially indistinguishable from the parental cell line. These cells have been used to establish an orthotopic xenograft model of localized prostate cancer expressing the wild type androgen receptor that responds to Casodex treatment in doses that are equivalent to those produced the 150 mg Casodex. We have demonstrated that implantation of Casodex (50mg sustained release 90 day pellets) into androgen replete nude mice induces significant

tumor regression, through cell cycle arrest and apoptosis, and induces significant changes in angiogenesis in the primary tumor. When grown as orthotopic tumors however, these cells do not appear to metastasize after treatment with Casodex. Using Laser Capture Micro-dissection (LCM) and Fluorescence Activated Cell Sorting (FACS) to purify the PC-346C^{RFP} cells from tumors. Using reverse transcriptase polymerase chain reaction (RT-PCR), Western analysis and immunofluorescence we have shown that the expression of Red Fluorescent Protein (RFP) is unaffected by treatment with Casodex (data not shown). Furthermore, even though the tumors undergo significant regression, the expression of the AR in the remaining tumor is essentially unaffected, either in its level or nuclear localization. This is in marked contrast to the in vitro data that has shown that expression of the AR is decreased in both LNCaP and PC-346C cells after treatment with Casodex and the receptor is relocalized to the cytoplasm (Lee et al., 2003).

Task 3b: To determine whether the presence of high levels in the Matrigel used for inoculation affect the metastatic progression of the PC-346C^{RFP} tumors, we have implanted PC-346C^{RFP} cells into the prostate of nude mice in growth factor deleted Matrigel. The PC-346C^{RFP} cell line has a wild type androgen receptor that does not bind to DHEA, obviating the problems with the mutant androgen receptor in the LNCaP. The growth of these cell lines in androgen supplemented nude mice is virtually identical to the growth of the cells in growth factor replete Matrigel, and these tumors also respond to treatment with Casodex (50mg sustained released 90 day pellets), regressing more rapidly tumors established with growth factor replete Matrigel, and showing signs

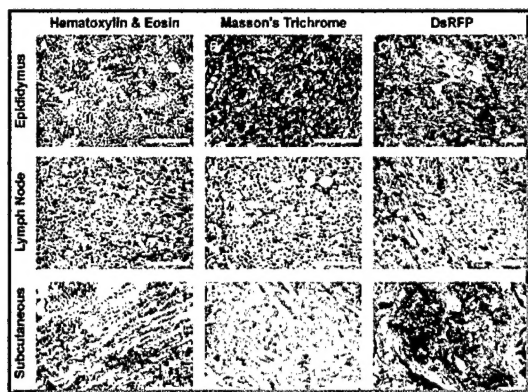


Figure 2: Morphology of metastatic deposits expressing Red Fluorescent Protein(DsRFP).

of metastatic progression to the lymph nodes, epididymis and subcutaneous sites, as evidenced by the presence of RFP staining in the metastatic deposits (Figure 2). This pilot study utilized 5 animals for each of the experimental groups, and even though 3/5 animals developed metastases in one or more sites was not large enough to reach statistical significance. This experiment is being repeated with the same experimental design using 15 animals per group, and several time points to ensure robust analysis.

The manuscript, co-authored with our colleagues at Erasmus University and appended to last year's annual report will not be submitted until these data are compiled and included they represent a major new component to the development of the model system.

Task 4: We have established the protocols needed for the preparation of RNA for gene array from samples prepared from frozen orthotopic tumors before and after treatment with Casodex. To facilitate these experiments and eliminate variability due to tumor composition which would confound the data analysis, we have developed a very efficient methodology for isolating the human prostate cancer cells from the primary tumor (where they may be contaminated with host stroma), and from metastatic sites (where they may be contaminated with both host stroma and epithelium). This methodology is based on Fluorescence Activated Cell Sorting (FACS) as outlined in Figure 3. This involves dicing the tumors into 1 mm fragments and incubated with CTC (1% collagenase, 0.1% trypsin and 1% chicken serum to dissociated the epithelial cells of the tumor

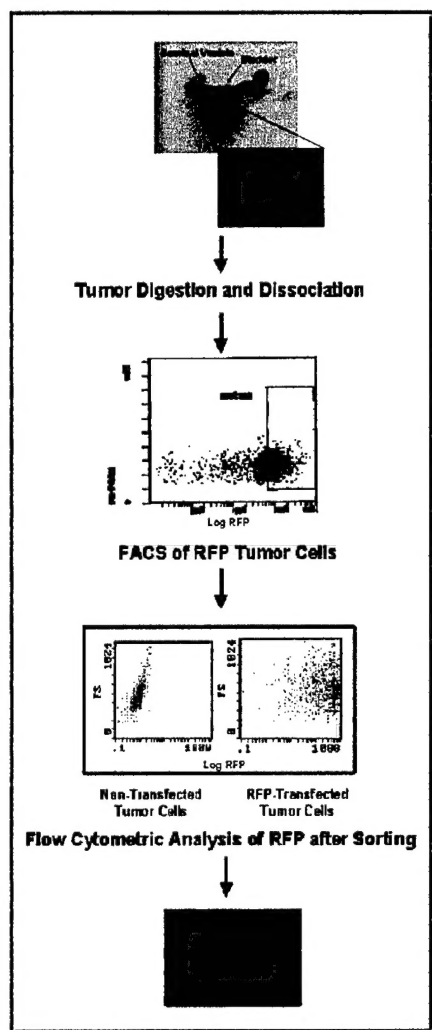


Figure 3: FACS-based purification of PC-346C^{RFP} cells.

(Montpetit and Tenniswood, 1989), prior to cell sorting on a Beckman-Coulter ALTRA FACS. As outlined in Fig. 3, this methodology has been used to purify PC-346C^{RFP} cells to greater than 98% purity, a purity that is suitable for planned gene array studies. During the course of a 2 hour sort, >500,000 PC-346C^{RFP} positive cells can be purified, providing enough material for RNA and Western analysis (cf data in Figure 2).

To standardize the gene arrays we have used RNA isolated from PC-346C cells treated with Casodex *in vitro*. These samples have been analyzed by Microarray (contracted with NimbleGen Systems, Madison, WI) and through a selective nylon based macroarray using a small subset of candidate genes associated with apoptosis and metastasis. The latter arrays identified functional changes in gene expression using a novel search paradigm based on comparative protein functionality and expression we have recently submitted for publication (Chrenek et al., 2004, appendix 5). As shown in Table 1, the steady state levels of a number of RNA transcripts are modulated by Casodex. The selection of these genes from the full array is very restrictive since the selection criteria require that the gene be upregulated in 9/9 arrays, and must also be induced in MCF-7 cells after treatment with anti-estrogens. The purpose of these stringent criteria is to focus on genes that are commonly involved in multiple models of apoptosis, and therefore likely to be involved in the central pathway(s) of apoptosis. The steady state mRNA levels of a number of other genes which are thought to be involved in regulating apoptosis, including clusterin, embigin, members of the bcl2 family (both pro- and anti-apoptotic members), p53 and mdm2 are not changed in this short term *in vitro* experiment.

The data from the first Nimblegen arrays is being analyzed now, and this will allow us to customize an array of approximately 400 pertinent genes for the complete microarray analysis of the changes in gene expression induced by Casodex in the primary tumors. Since these customized arrays cost about 5% of the complete microarrays, the cost of these experiments is not prohibitive. The changes that we have noted from the nylon macroarray is now being validated by Northern analysis, RT-PCR or Western analysis, using tissue samples derived from both FACS based purification and LCM based purification, to establish that the isolation methodology does not bias the results

Spot number	Gene	Fold reduction by Casodex (2 fold or more)	Fold Induction by Casodex (2 fold or more)
1	Tumor necrosis factor receptor superfamily 1A (TNFR1A)	3.4	
2	Fas	2.1	
3	death associated protein kinase 1 (DAPK1)		4.7
4	mitogen activated protein kinases 7 and 8 (MAPK 7 and MAPK8)	4.8	
5	mitogen activated protein kinase kinase 6 (MAPKK 6)	5.4	
6	retinoblastoma-binding protein 3 (RBBP3),	2.17	
7	c-jun		3.3
8	Cdk2	2.1	
9	inhibitor of apoptosis protein-2 (IAP-2)		2.2
10	c-abl	12.5	
11	IGF-1		2.8
12	Caspase 4	9.0	

Table 2: Modulation of gene expression by Casodex in PC-346C^{RFP} cells *in vitro*

Key Research Accomplishments

- Characterization of cellular pathways involved in induction of apoptosis after anti-androgen therapy in PC-3465C^{RFP} cells *in vitro*, with particular emphasis on the role of the mitochondria (documented in Lee et al., 2003 and Zhan et al., 2002)
- Publication of two review papers germane to this project, both of which reference the central hypothesis being tested in the experiments outlined in this report (Lee and Tenniswood 2004a, 2004b).
- Demonstration that invasive sublines of LNCaP cells are not highly metastatic *in vivo*, (probably due to the high levels of adrenal steroid, DHEA, in the rodent host).
- Establishment and refinement of new model of androgen dependent anti-androgen responsive localized prostate cancer
- Demonstration that treatment of orthotopic tumors derived from PC-346C^{RFP} cells induce apoptosis in response to Casodex
- Demonstration that Casodex treatment can also induce metastatic progression in the PC-346C^{RFP} cells, providing support for the suggestion that the LNCaP cell line and its derivatives may not be ideal model cell lines for orthotopic studies, and providing a possible explanation for the failure of the invasive LNCaP cells to metastasize.
- Development of a robust methodology for the isolation of RFP tagged cells from orthotopic tumors (primary and metastatic) that can be used for Gene array analysis and Western analysis.
- Macroarray identification of < 1.5 fold changes in several genes involved in mitosis, apoptosis or metastasis.

Reportable Outcomes

We have created a number of novel cell lines including:

- PC346C^{RFP}
- LNCaP^{RFP}
- PC346C^{GFP}
- DU-145^{RFP}
- DU-145^{GFP}

Since the last annual report a total of 5 manuscripts have been published (2), are in press (2) or have been submitted (1), all of which acknowledge the support of DAMD17-01-1-0114:

Zhan P., Lee, E.C.Y., Packman, K. and Tenniswood, M. (2002) Induction of Invasive Phenotype by Casodex in hormone sensitive Prostate Cancer Cells. *Journal of Steroid Biochemistry and Molecular Biology* 83: 101-111.

Lee, E.C.Y., Zhan, P., Packman, K., and Tenniswood, M. (2003) Anti-androgen induced cell death in LNCaP Human Prostate Cancer Cells. *Cell Death and Differentiation* 10:761-771.

Lee, E.C.Y. and Tenniswood, M. (2004) Programmed Cell Death and Survival Pathways in Prostate Cancer Cells. *Archives of Andrology* 50:27-32.

Lee, E.C.Y. and Tenniswood, M. (2004) Emergence of Metastatic Hormone Refractory Disease in Prostate Cancer after Anti-androgen Therapy. *Journal of Cellular Biochemistry* 91: (in press, March Issue)

Chrenek, M., Erickson, T., Gee, C. Lee, E.C.Y. Gilmore K., Tenniswood, M., and Wong P. (2004) Comparative Functional Genomics: Analysis of Changes in mRNA Profiles in Multiple Model Systems for Understanding Basic Biological Phenomenon. *Transactions of the Integrated Biomedical Informatics and Enabling Technologies* 1: (in press) [TIBETS is a new Web-based open access journal].

In addition we have completed the following manuscript which has been sent to our collaborators in Erasmus University for their approval:

Lee, E.C.Y., Ayala, G., Flanagan, L., Packman K., Van Weerden, W., Romijn, J. and Tenniswood, M. (2003) Characterization of Casodex-Responsive Orthotopic Xenografts of Androgen Receptor Positive PC-346C^{RFP} Human Prostate Carcinoma Cells. (to be submitted to *Urology*). However as pointed out in the Body of this report, we are waiting for the results from the experiments in which the cells have been inoculated in growth factor depleted Matrigel, since these findings may considerably improve the responsiveness of the tumors to anti-androgens or other manipulations.

Conclusions

The experiments completed to date provide in vitro support for the hypothesis that treatment of localized prostate cancer with Casodex or use of the drug in a chemo-preventive setting, may be inappropriate and lead to a higher tumor burden of androgen independent metastases following treatment. *In vivo* orthotopic xenograft modeling of tumor progression in nude mice will be required to further substantiate this hypothesis.

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APPENDIX ONE



Induction of invasive phenotype by Casodex in hormone-sensitive prostate cancer cells[☆]

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Abstract

The cellular mechanisms of anti-androgen-induced tumor regression have not been investigated in great detail. We have compared the induction of cell death in the androgen-dependent, non-invasive LNCaP prostate cancer cell line by Casodex and TNF- α . Both agents induce a dose and time-dependent decrease in cell viability in vitro. However, Casodex does not induce classical DNA fragmentation to oligonucleosomes typically induced by TNF- α , but rather induces cleavage to form intermediate 60 kb DNA fragments. RT-PCR based analysis demonstrates that in LNCaP cells Casodex coordinately alters the expression of steady-state level of mRNAs of several matrix metalloproteases and their cognate inhibitors (most notably MMP2 and TIMP1). Zymography and reverse zymography confirm that the ratio of metalloprotease(s) to inhibitor(s) is altered in favor of activation of the proteases. In a small percentage of the treated LNCaP cells, the activation of the extracellular matrix (ECM)-proteases by Casodex also induces an invasive phenotype. The acquisition of an invasive phenotype is not seen when LNCaP cells are treated with TNF- α , and is not seen when the LNCaP cells are treated with both compounds simultaneously, suggesting that the phenomenon may be specific to particular classes of compounds. These observations have significant implications in the treatment of prostate cancer, since the appearance of a more aggressive phenotype following treatment is clearly undesirable.

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Keywords: Casodex; Prostate cancer; LNCaP; Metastasis; Tumor invasion

1. Introduction

In 1999, an estimated 180,000 men were diagnosed with prostate cancer in the United States, and 37,000 succumbed to the disease. The main cause of death lies in the invasion and metastasis of prostate cancer, although for many the disease will remain localized and slow growing. More extensive PSA screening has lead to the identification of many more early stage (A1 and A2) tumors. Approximately 70% of these tumors are indolent and will not need treatment during the patient's life time, however, at the present time there is no way to distinguish between the aggressive, clinically significant tumors that need to be treated and indolent tumors. The lack of histological or biochemical markers to predict which tumors have the potential to progress to a metastatic phenotype, has resulted in many patients being treated more aggressively than is necessary. While standard treatments for early stage disease are surgery or radiation therapy, an increasing number of patients are be-

ing treated with anti-androgens, particularly flutamide and Casodex. Furthermore, neo-adjuvant therapy using these anti-androgens to debulk organ-confined prostate tumors (particularly stage B1) and to improve positive margins is now widely used prior to surgery. In addition, many patients at high risk for prostate cancer are considering chemoprevention in the form of anti-androgens. With this increased usage of anti-androgen, it is important to fully characterize the effects of anti-androgens on localized tumors.

Anti-androgens induce prostate tumor regression by initiating activate cell death, or apoptosis [1,2]. Apoptosis is a type of programmed cell death that requires both RNA and protein synthesis [3,4] to maintain tissue homeostasis and proper disposal of cells. In glandular epithelia such as the prostate, the process of apoptosis can be broken down into several distinct steps [5]. During the pre-condensation stage, most of the genes that are necessary for cell death are induced de novo, while other necessary proteins are recruited from other functions in the gland. In the cytoplasmic condensation stage, the dying cell loses interaction with its neighbors as the extracellular matrix (ECM) is degraded and the cytoplasmic volume decreases. This is followed by the nuclear condensation phase during which endonucleases are activated resulting in the fragmentation of the DNA, producing the hyperchromatic, pyknotic nucleus

[☆] Proceedings of the 15th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology, "Recent Advances in Steroid Biochemistry and Molecular Biology", Munich, Germany, 17–20 May 2002.

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characteristic of apoptotic cells. During the fragmentation phase, the apoptotic cell is subdivided into several apoptotic bodies which are phagocytosed by the neighboring epithelial cells or macrophages.

In the pre-condensation phase, two major pathways have been identified for the induction of apoptosis. The first is initiated by withdrawal of growth factors, for example, removal of trophic hormones in the prostate. The second involves signaling through death-receptors on the cell surface, such as TNF-RI and Fas. In this case, apoptosis is induced by receptor ligands such as TNF- α mediated cell death of lymphocytes. Regardless of which specific pathway initiates the apoptotic process, the intracellular "effector phase" and final execution phase of apoptosis appear to utilize common pathways, that are regulated by the Bcl-2 and caspase families [6]. The mitochondrion is also a critical regulatory component in many, if not all, forms of the apoptotic effector pathway. Accumulating evidence indicates that a transition in mitochondrial permeability is a critical event, or "central executioner" that integrates signaling from different inductive stimuli and orchestrates the executioner phase of apoptosis [7].

Many prostate epithelial cells and tumor cells are responsive to TNF- α and/or Fas and utilize this death-receptor mediated pathway to initiate cell death. Signaling through the TNF- α receptor leads to the autocatalytic activation of caspases which initiates a proteolytic cascade that leads to mitochondrial permeability transition (PT), releasing cytochrome *c* into the cytoplasm. The release of cytochrome *c* from the mitochondria is blocked by Bcl-2 which appears to associate with the outer mitochondrial membrane, attenuating mitochondrial PT [8]. Other Bcl-2 family members, including Bax and Bad, attenuate the protective function of Bcl-2. While the expression of Bcl-2 and its dimerization partners has not been studied in the normal prostate in detail, it is known that Bcl-2 is upregulated in hormone refractory prostate tumors [9], suggesting that the aberrant expression of this protein may contribute to inappropriate survival of tumor cells.

Cytoplasmic condensation requires the activation of a number of ECM-proteases including matrix metalloproteases (MMP2 and MMP9) which are expressed in dying prostatic epithelial cells [10–14]. The activity of the proteases is regulated by specific inhibitors including the tissue inhibitors of metalloproteases (TIMP1 and TIMP2) and when activated, the extracellular proteolytic cascade overrides the inhibition and results in the degradation of many of the components of the basement membrane. Since the degradation of the basement membrane is a prerequisite for the cytoplasmic condensation of the dying cell, induction and activation of secreted proteases is a necessary part of apoptosis of secretory epithelial cells. In glandular tissues such as the prostate, changes in ECM and the consequent loss of cell–cell and cell–substratum interactions appear to be co-incident with the cleavage of actin and precede DNA fragmentation which occurs during nuclear

condensation [15,16]. DNA cleavage, which can be monitored by agarose gel electrophoresis or in situ end labeling (ISEL or TUNEL labeling), is thought to be downstream of caspase activation [17,18] and requires the activation of Ca²⁺/Mg²⁺-dependent endonuclease(s) [19]. However, intranucleosomal DNA cleavage is not seen in all cell types undergoing apoptosis, and is clearly not necessary for apoptotic cell death. Furthermore, comparative studies using pulse field gel electrophoresis (PFGE) have shown that many cells undergoing apoptosis do not completely fragment their DNA, even though the enzymatic activities necessary to do so are present in isolated nuclei [19]. This demonstrates that, although all cells appear to have the enzymatic apparatus necessary for intranucleosomal DNA fragmentation, subtle differences in chromatin structure, intranuclear pH, or activating divalent cation (Ca²⁺ and Mg²⁺) or inhibiting ion (Zn²⁺ and K⁺) concentrations may significantly alter the extent of DNA fragmentation, and may even block it entirely [20].

Abrogation of DNA fragmentation may result from a number of genetic or epigenetic alterations. For example, abrogation of the caspase cascade, due to alterations in the steady-state levels of members of the Bcl-2 family that disrupt the ratio of Bcl-2 and Bax, may block the cleavage of DNA Fragmentation Factor (DFF) and DNA fragmentation. It is also possible that subtle changes in the nuclear concentrations of specific ions, such as K⁺ and Ca²⁺ may also influence the activity of the endonucleases [20]. Alternatively, subtle changes in the ECM and/or growth factor micro-environment may play a critical role in determining the sensitivity of the cell to the induction of apoptosis following hormone ablation. In vitro, anti-androgens induce apoptosis in a large percentage of hormone-dependent cells, however, a small population fail to die and cease to require androgens for cell survival. While this phenomenon is often dismissed as an artifact of cell culture, it does reflect the clinical progression of prostate cancer, where resistance to anti-androgens eventually develops in nearly all patients treated with anti-androgen therapy. It has been suggested that resistance to anti-androgens is simply an outgrowth of "natural selection", where a small number of hormone-independent cells in a hormone-dependent tumor emerge. However, recent data suggests that the acquisition of hormone resistance may be due to the direct action of anti-androgens on hormone sensitive cells that alters the cell–substratum interactions without directly inducing mitochondrial permeability and cell death (Lee et al., unpublished data).

2. Experimental procedures

2.1. Cell culture

LNCaP, an androgen receptor positive and non-invasive prostate cancer cell line (ATCC, Rockville, MD) were

maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) (Sigma), 100 unit/ml penicillin, and 100 µg/ml streptomycin. Prior to each experiment, LNCaP cells were plated in RPMI-1640 supplemented with 10% FBS, and after 24 h transferred to a serum-free growth controlled (GC) medium (RPMI-1640, 2 mg/ml BSA-V, 1 ng/ml EGF, 0.5 mg/ml fetuin, 50 nM hydrocortisone, 20 µg/ml insulin, 25 nM sodium selenite, 0.5 mM sodium pyruvate, 0.1 nM T₃, 10 µg/ml transferring, 1 nM testosterone) for an additional 24 h prior to treatment. Growth of LNCaP cells in this medium mimics the cell kinetics of prostate epithelial tumor cells grown *in vivo*. When LNCaP cells were pre-treated with synthetic androgen R1881 (Methyltrienolone) (NEN Life Science Products, Inc., Boston, MA), androgens were added in GC medium for 24 h prior to other treatments. Cells were pre-treated with caspase inhibitors for 1 h in GC media and the cells were washed with PBS prior to the addition of Casodex or TNF-α in GC medium. Cell viability was assayed by MTT assay (Sigma).

2.2. DNA fragmentation analysis

Low molecular weight DNA was isolated from LNCaP cells treated with control vehicle, Casodex or TNF-α using standard protocols, dissolved in TE buffer and run on 2% agarose gel electrophoresis at 60 V for 2 h followed by 0.1 µg/ml ethidium bromide staining.

2.3. Flow cytometry

For analysis of cell cycle kinetics, LNCaP cells treated with control vehicle, Casodex or TNF-α were harvested by trypsinization, fixed with 90% ethanol and incubated with 2.5 mM EDTA, 50 µg/ml propidium iodide and 1 U/ml RNase in phosphate buffer saline (PBS) for 30 min at room temperature. For analysis of DNA fragmentation, LNCaP cells were treated with 10 ng/ml TNF-α or 100 µM Casodex were harvested by trypsinization,

fixed with 2% formaldehyde and permeabilized with 70% ethanol. 3'-OH DNA ends were labeled with Terminal Transferase kit (Roche, Indianapolis, IN) and detected with Br-dUTP staining kit (Phoenix Flow System, San Diego, CA). All samples were analyzed using an EPICS XL Flow Cytometer and were modeled with the Multiplus AV software (Phoenix Flow Systems).

2.4. Invasion assay

Transwell inserts with 8 µm pore size were coated with 35 µg/cm² growth factor-reduced Matrigel (BD Biosciences, Bedford, MA). Surviving adherent cells after treatment with Casodex or TNF-α were plated in transwell inserts in GC medium. Fibroblast conditioned-medium was placed in the lower chamber. Cells were incubated for 48 h, fixed with glutaraldehyde, and invasive cells on the underside of the transwell membrane well were stained with crystal violet (Sigma) and counted.

2.5. Semi-quantitative RT-PCR analysis

Total mRNA was extracted from treated LNCaP cells using Tri-ReagentTM. cDNA was prepared from total mRNA using a cDNA synthesis kit (Gibco Life Technologies, Rockville, MD). Oligonucleotide primers were designed using PRIMER DESIGNERTM version 2.0 software (Scientific and Educational Software) and synthesis by Molecular Core Facility of Adirondack Biomedical Research Institute (ABRI) or Biocore Facility of University of Notre Dame. The PCR amplification condition for each gene was optimized using OptimizerTM kit (Invitrogen). PCR product was confirmed by sequencing at the molecular core facility at ABRI. Quantification of mRNA level was analyzed using SigmaGelTM (Sigma), followed by densitometric scanning.

2.6. Zymography and reverse zymography analysis

For analysis of protease and protease inhibitor activities, zymography and reverse zymography, LNCaP cells were

Gene	Primers	PCR products (bp)
Cathepsin B	Sense: 5'-CAACTCCTGGAACACTGACT-3' Anti-sense: 5'-GAAGGCGAAGAAGCTGCAACAC-3'	262
MMP2	Sense: 5'-GGACAGATGGATACAGATGG-3' Anti-sense: 5'-GTCCTCGGAGTGCTCTAATC-3'	319
MMP9	Sense: 5'-TACTCTGCCTGCACCACTAA-3' Anti-sense: 5'-CAGTGTCTGAAGTTCGATGTG-3'	249
TIMP1	Sense: 5'-ATCCTGTTGTTGCTGTGGCTGATG-3' Anti-sense: 5'-TGCTGGGTGGTAACCTCTTTATTCA-3'	667
TIMP2	Sense: 5'-AAACGACATTTATGGCAACCCTATC-3' Anti-sense: 5'-ACAGGAGCCGTCACTTCTCTTGATG-3'	405
VEGF	Sense: 5'-CCTGGTGGACATCTTCCAGGAGTACC-3' Anti-sense: 5'-CTCACCGCCTCGGCTTGTC-3'	275, 407, 497, 530

Oligonucleotide primers for RT-PCR analysis of various genes.

treated with 0, 50 or 100 μM Casodex in GC medium minus BSA for 72 h. After treatment, media were collected and concentrated to 50 μl by centrifugation using Centricon filter (NMWL; Millipore) at $3000 \times g$ for 2 h. Proteins from concentrated media (5 or 10 μl) were separated on zymogram gels (0.04 mg/ml gelatin, 7.5% acrylamide/bis-acrylamide, 375 mM Tris-HCl (pH 8.8), 0.1% SDS, 0.1% ammonium persulfate and 0.002% TEMED) at 4°C at 8 mA for 5 h, soaked twice in Triton X-100 wash buffer (50 mM Tris-HCl (pH 7.4), 2.5% Triton X-100, 0.05% azide) for 30 min and incubated at 37°C for 16 h with shaking in Ca^{2+} buffer (50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 10 mM CaCl_2 , 0.05% azide). Gels were then stained in zymography Coomassie blue solution (0.005% Coomassie blue R-250, 10% acetic acid (v/v), 10% isopropanol (v/v)). For reverse zymography analysis, proteins were separated on 15% SDS-PAGE gels with 1 ng/ml gelatinase B and stained as described above.

2.7. Statistical analysis

Statistical comparisons were performed using the Kruskal-Wallis ANOVA for multiple groups. Post-test comparisons between groups were made using the Dunn test. Differences between means were considered significant when P -values less than 0.05 were obtained. All computations were performed using the GraphPad Instate statistical program (Intuitive Software for Science, San Diego, CA). Data are expressed as mean \pm standard deviation.

3. Results

3.1. LNCaP cells are quiescent grown in GC serum-free defined medium

To develop an in vitro cell culture model that mimics the remarkably low cell proliferation rate in both normal prostate and in localized and metastatic tumors in vivo, LNCaP cells were cultured in a serum-free medium supplemented with specific growth factors. The cell cycle kinetics of LNCaP cells grown in various culture conditions at different times were determined by flow cytometric analysis with propidium iodide (PI) staining. As shown in Table 1, LNCaP cells grown in RPMI medium supplemented with 10% of FBS typically show a 20% proliferation rate, as indicated by the percentage of S phase cells. Twenty-four hours after shifting these cells to GC serum-free medium, the percentage of S phase cells decreases to 10% both in the absence and presence of 1 nM testosterone, suggesting that testosterone does not affect cell cycle kinetics of LNCaP cells grown under these conditions. In contrast, the cells maintained in RPMI-1640 with 10% FBS continued to proliferate ($S = 17\%$) (data not shown). The percentage of cells in S phase continues to decrease upto 72 h in GC medium at which point only 2% of the cells are in S phase, indicating that

Table 1
Cell cycle kinetics of LNCaP cells under different culture conditions

Culture conditions		Percentage at different phases of cell cycle		
Media	Time (h)	G ₀ /G ₁	S	G ₂ /M
RPMI-1640	-24	68.5	20.6	9.9
GC	0	68.2	18.6	13.2
GC + T	24	78.6 \pm 4.6	10.3 \pm 3.2	11.1 \pm 2.4
GC	24	79.2 \pm 3.4	10.1 \pm 0.4	10.8 \pm 3.0
GC	72	88.2 \pm 1.4	2.4 \pm 1.0	9.4 \pm 0.4
GC + T	72	86.5 \pm 0.8	3.9	9.6 \pm 0.8

LNCaP cells were plated in RPMI-1640 supplemented with 10% FBS and grown to approximately 70% confluency. At this time point (-24 h), the medium was replaced with serum-free GC medium in the absence or presence of 1 nM testosterone for 24 or 72 h. Cell cycle expressed as mean \pm S.D. were measured by flow cytometry as described in section 2.

LNCaP cells essentially become mitotically quiescent under these conditions.

3.2. Casodex induces cell death in LNCaP cells

Casodex is designed to reduce tumor size by binding to both normal and mutated androgen receptor and acting as an antagonist. To investigate the effect of Casodex on the viability of LNCaP cell culture model, cells grown in GC medium were treated with 0, 10, 50 or 100 μM Casodex for 24, 48 or 72 h (Fig. 1). Cell viability of adherent LNCaP cells measured by MTT assay demonstrates that Casodex induces the loss of mitochondrial dehydrogenase activity and cell death in LNCaP cells in a time and

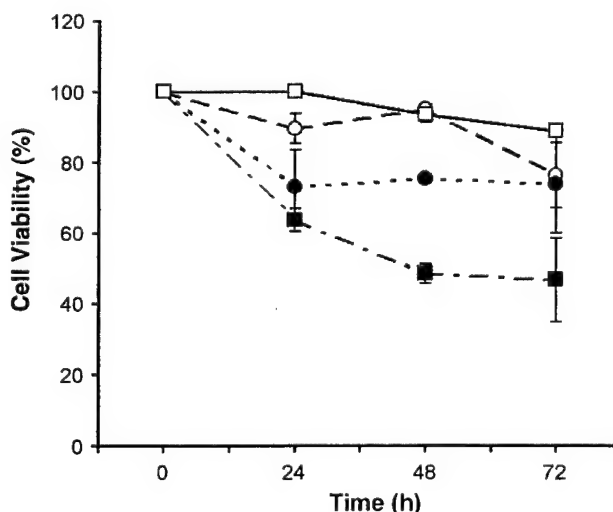


Fig. 1. Time course and dose response of cell death of LNCaP cells after Casodex treatment. LNCaP cells were treated with 0 (\square), 10 (\circ), 50 (\bullet) and 100 μM (\blacksquare) Casodex for 24, 48 and 72 h in GC medium. Cell viability of adherent cells measured by MTT assay is expressed as percentage of viable cells compared to untreated controls. Results are expressed as mean \pm S.D.

dose-dependent manner. In particular, Casodex induces cell death in 50% of LNCaP cells by 48 h in GC medium at the concentration of 100 μ M, which is approximately the effective steady-state serum level of Casodex achieved in clinical trials with locally advanced prostate cancer in which patients received 150 mg Casodex once per day ($C_{ss} \approx 27 \mu\text{g/ml}$ (90 μM)).

3.3. Casodex-induced limited DNA Fragmentation in LNCaP cells

Formation of nucleosomal DNA ladders is one of the characteristic features of apoptotic cell death. TNF- α induces apoptosis in LNCaP cells and is well known to induce the formation of oligonucleosomal ladders. As shown in Fig. 2

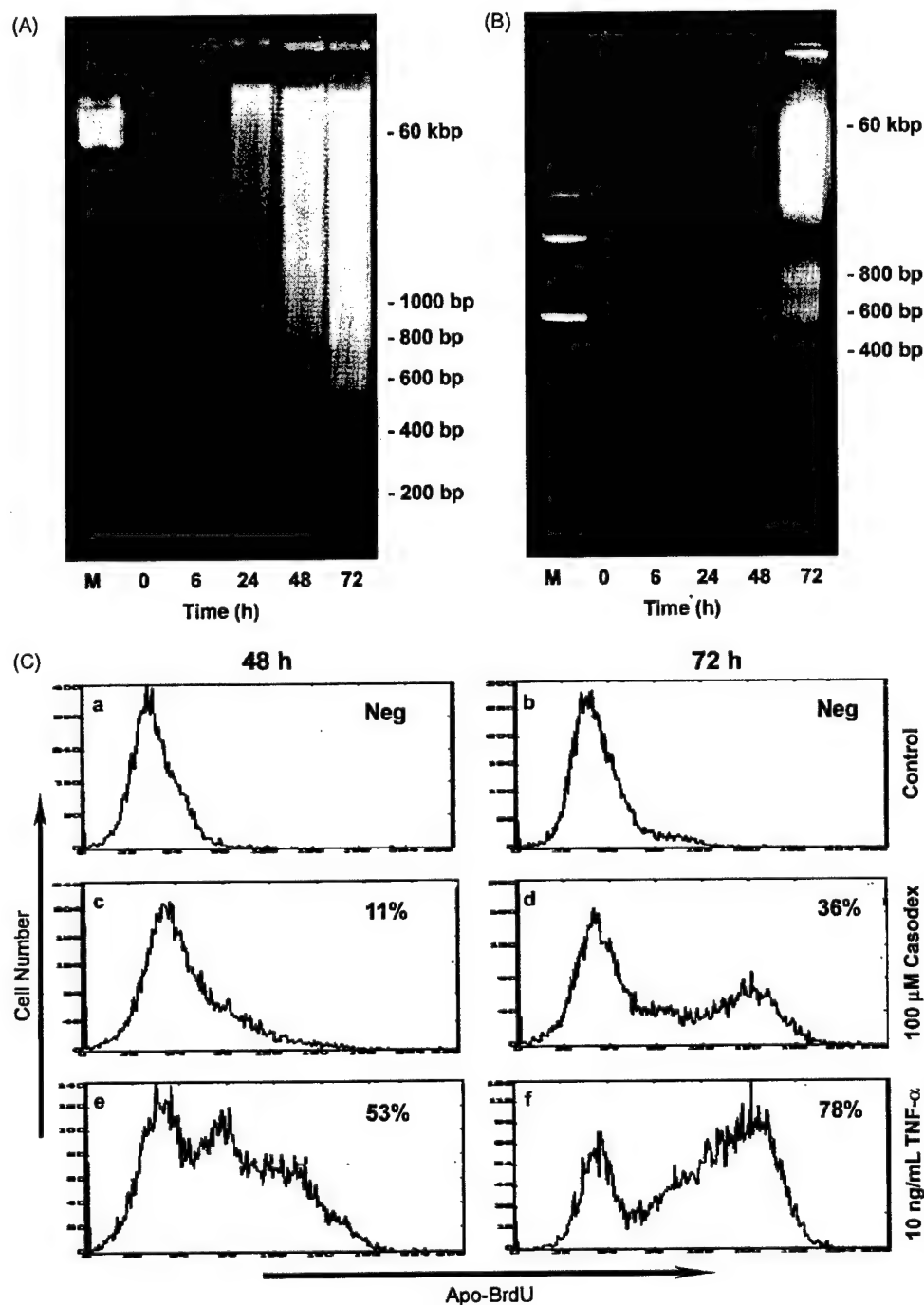


Fig. 2. DNA fragmentation in LNCaP cells treated with TNF- α or Casodex. Low molecular weight DNA isolated from LNCaP cells treated with 10 $\mu\text{g/ml}$ TNF- α (Panel A) or 100 μM Casodex (Panel B) for 6, 24, 48 or 72 h in GC medium was electrophoresed on 2% agarose gel and stained with ethidium bromide. (Panel C) LNCaP cells were treated with control vehicle (a and b), 10 μM Casodex (c and d), 100 μM Casodex (e and f) or 10 ng/ml TNF- α (g and h) for 48 h (a, c, e) or 72 h (b, d, f). DNA fragmentation was measured by BrdU staining and analyzed by flow cytometry as described in section 2. Percentage of cell death for each treatment is indicated in upper right quadrant of each panel.

(Panel A), after treatment with 10 ng/ml TNF- α , LNCaP cells show clear evidence of oligonucleosomal ladder formation between 6 and 24 h, and show increasing evidence of DNA ladder formation over 72 h. In contrast, when LNCaP cells were treated with 100 μ M Casodex, DNA cleavage does not become evident until 48 h of treatment (Fig. 2, Panel B). Furthermore, while fragmentation to ~60 kbp is clearly evident at 72 h, there is minimal formation of the classical DNA oligonucleosome ladder. These data suggest that while Casodex induces DNA cleavage, it is not the classical cleavage associated with apoptosis, and is significantly delayed compared to that seen with TNF- α . To quantitatively analyze apoptotic cell death, DNA fragmentation was measured using Apo-BrdU staining and analyzed by flow cytometry. After treatment with 10 ng/ml TNF- α for 48 and 72 h, there is a substantial increase in the proportion of cells (53 and 78%, respectively) labeled by Apo-BrdU (Fig. 2, Panel C, e and f). In contrast, only 11 and 35% of LNCaP cells show signs of DNA fragmentation after treatment with 100 μ M Casodex for 48 and 72 h, respectively (Fig. 2, Panel C, c and d), in agreement with the agarose gel DNA fragmentation analysis (Fig. 2, Panels A and B).

3.4. Casodex-induced apoptotic cell death is an AR-dependent event

The anti-androgenic properties of Casodex are thought to be elicited through the binding of the androgen receptor. To establish that effects of Casodex are mediated through its interaction with the receptor, LNCaP cells were treated with Casodex in the absence or presence of 1 nM R1881, a synthetic androgen that is metabolized very slowly in cell culture, and has a very high affinity for the androgen receptor. It thus serves as a chronic agonist of androgen receptor mediated actions. LNCaP cells were pre-treated with R1881 for 24 h before treatment with 100 μ M Casodex or 10 ng/ml TNF- α for 72 h. When cells were treated with 100 μ M Casodex, there is a significant increase of viable cells in R1881 pre-treated LNCaP cells as measured by the MTT assay (Fig. 3, Panel A). As demonstrated by the decrease in Apo-BrdU incorporation, R1881 significantly reduces the induction of cell death by Casodex from 48 to 8%, rendering the cells almost resistant to Casodex over 72 h time interval (Fig. 3, Panel B). On the other hand, R1881 does not affect the induction of cell death by TNF- α (data not shown). These results demonstrate that Casodex induces cell death through an androgen receptor mediated mechanism.

3.5. Acquisition of invasive potential in LNCaP cells after Casodex treatment but not TNF- α treatment

Prostate cancer patients treated with anti-androgens ultimately become hormone refractory and have an increased propensity for metastasis. To assess invasive potential of LNCaP cells both before and after treatment with Casodex or TNF- α , Boyden chamber invasion assays were performed.

Casodex induces an increase in the number of invasive cells in a dose-dependent manner in both the presence and absence of 1 nM testosterone (Fig. 4, Panel A). TNF- α , on the other hand, does not produce an increase in invasive potential of the surviving LNCaP cells after treatment (Fig. 4, Panel B). In fact, at the doses that produce similar or greater levels of cell death (Fig. 5, Panel A), TNF- α does not, on its own, increase the invasive potential of LNCaP, but is able to attenuate Casodex-induced increase in invasive potential of surviving cells (Fig. 5, Panel B). Morphological assessment of the invasive cells that survive Casodex treatment suggests that the cells have acquired different morphologies than the cells that survive TNF- α treatment (Fig. 4, Panel C). LNCaP cells that survive Casodex treatment displayed a neuronal-like and stellate morphology, characteristic of many invasive cell lines. In contrast, cells surviving TNF- α treatment display no discernible difference in morphology from control cells. These data together demonstrate that while Casodex, at clinically relevant doses, induces cell death in LNCaP cells *in vitro*, it also appears to induce an invasive phenotype in a small proportion of the surviving cells (approximately 80/20,000 cells, 0.4%).

3.6. Expression of genes that are associated with tumor invasion and metastasis are altered after Casodex treatment

ECM-proteases, which is a large gene family composed of several members, have been identified as key players in tumor invasion and metastasis. To understand the dysregulation of these important components as the cell death process is initiated, the change of expression of these genes after treatment with Casodex or TNF- α in LNCaP cells were examined by semi-quantitative RT-PCR. As shown in Fig. 6, Panels A and B, the steady-state level of MMP2 mRNA is enhanced 2-fold after 10 μ M Casodex treatment for 72 h and a similar enhancement of MMP2 expression after TNF- α treatment is also seen. The steady-state level of TIMP2, the cognate inhibitor of MMP2, is not changed by either Casodex or TNF- α treatment. Whereas MMP9 mRNA is not detectable in LNCaP cells and is not induced after treatment with either Casodex or TNF- α , these treatments dramatically reduces the steady-state level of TIMP1 mRNA level by 4- and 1.5-fold, respectively, after 72 h, as quantified by densitometric scanning (Fig. 6, Panel C). The decrease of TIMP1 expression is time and dose-dependent to Casodex treatment, which parallels the decrease of number of viable cells within the whole cell population, suggesting that change of TIMP1 level corresponds to cell death process. To confirm that the changes in the steady-state mRNA level are reflected by changes in enzyme activity, the metalloprotease activities of MMP2 and MMP9 were assayed by zymography and the inhibitor activities of TIMP1 and TIMP2 were assessed by reverse zymography. The activity of MMP2 (72 kDa) is significantly upregulated after 100 μ M Casodex treatment whereas the activity of MMP9 (92 kDa)

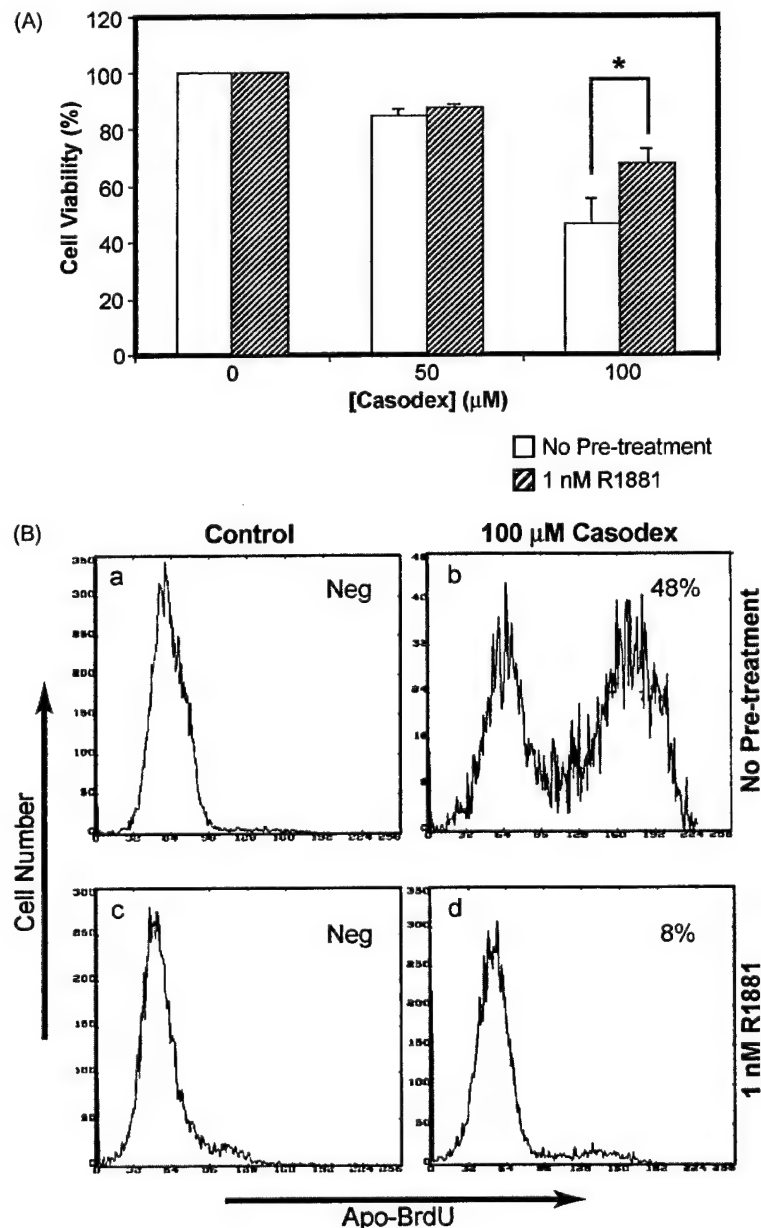


Fig. 3. Casodex-induced cell death is an AR-dependent event. (Panel A) LNCaP cell pre-treated in GC medium in the absence (*open*) or presence (*hatched*) of R1881 for 24 h were treated with 0, 50 or 100 μM Casodex for 72 h in GC medium. Cell viability measured by MTT assays is expressed as the percentage of viable cells compare to untreated controls (mean \pm S.D.), * P < 0.05. (Panel B) LNCaP cell pre-treated in GC medium in the absence (a, b) or presence (c, d) of R1881 for 24 h were treated with control vehicle or 100 μM Casodex for 72 h in GC medium. DNA fragmentation was measured by BrdU staining and analyzed by flow cytometry as described in section 2. Percentage of cell death of each treatment is indicated in upper right quadrant of each panel.

remains the same at the detectable level across the different conditions (Fig. 6, Panels D and E). The activity of TIMP1 (28 kDa) is downregulated after treatment with 50 μM Casodex as shown by reverse zymography (Fig. 6, Panels F and G) where it is barely detectable with 100 μM Casodex treatment. In contrast, TIMP2 (21 kDa) activity is clearly increased after Casodex treatment (Fig. 6, Panels F and G). The induction of expression of these genes is essential to enable the LNCaP cells to acquire invasive potential. The observation is a strong evidence to explain how the surviv-

ing LNCaP cells that migrate through the Matrigel membrane and grow on the other side, acquire invasive ability after treatment with Casodex.

Angiogenic factors have also been identified as major contributors in tumor invasion and metastatic progression. Steady-state levels of several isoforms of angiogenic factor VEGF increase in a dose-dependent fashion in LNCaP cells following treatment with Casodex or TNF- α (Fig. 7, Panel B). The increase is clearly in a time-dependent manner in Casodex treated cells, which results in a 6-fold induction at

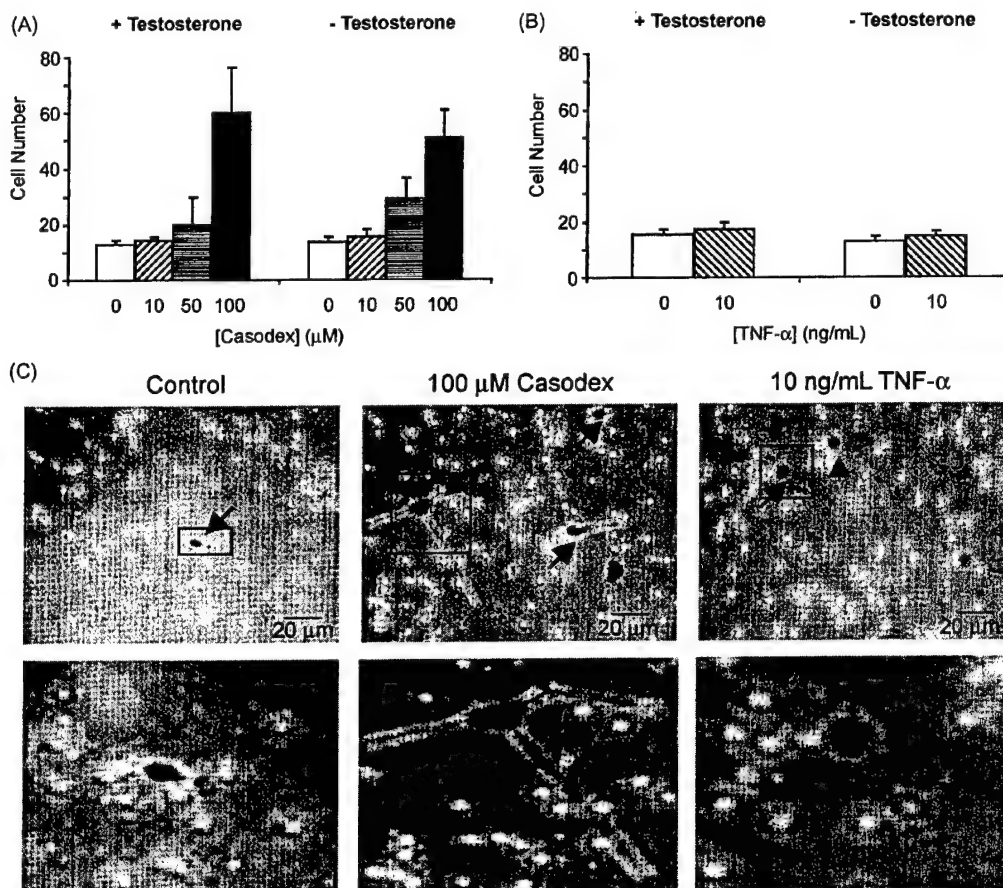


Fig. 4. LNCaP cells acquire invasive potential after Casodex treatment. LNCaP cells were treated with 0, 10, 50 or 100 μM Casodex (Panel A) or 0 or 10 ng/ml TNF-α (Panel B) in GC medium in the absence or presence of 1 nM testosterone for 72 h. Surviving adherent cells (20,000 cells) after treatment with Casodex or TNF-α were re-plated in 8.0 μm transwell inserts precoated with Matrigel in GC medium. Cells that invaded to the underside of the membrane were stained with crystal violet, counted from 10 randomly chosen fields. Results are expressed as mean ± S.D. (Panel C) Phase contrast photographs of invasive LNCaP cells after treatment with Casodex and TNF-α at 200× magnification (bar represents 20 μm). The bottom panel is enlargement of the corresponding fields outlined in the top panel.

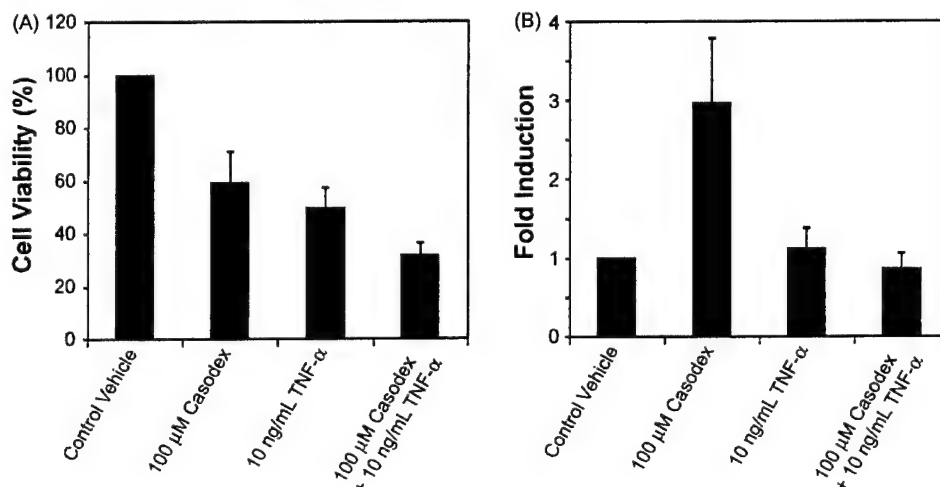


Fig. 5. Combined effect of Casodex and TNF-α on cell death and cell invasion of LNCaP cells. (Panel A) LNCaP cells were treated with 100 μM Casodex, 10 ng/ml TNF-α or 100 μM Casodex plus 10 ng/ml TNF-α for 72 h in GC medium. Cell viability measured by MTT assay was expressed as mean ± S.D. (Panel B) LNCaP cells were treated with control vehicle 100 μM Casodex, 10 ng/ml TNF-α or 100 μM Casodex plus 10 ng/ml TNF-α for 72 h in GC medium. Surviving adherent cells (20,000 cells) after treatment with Casodex or TNF-α were plated in 8.0 μm transwell inserts precoated with Matrigel in GC medium. Cells that invaded to the underside of the membrane were stained with crystal violet, counted and expressed as fold of induction as compare to controls. Results are expressed as mean ± S.D.

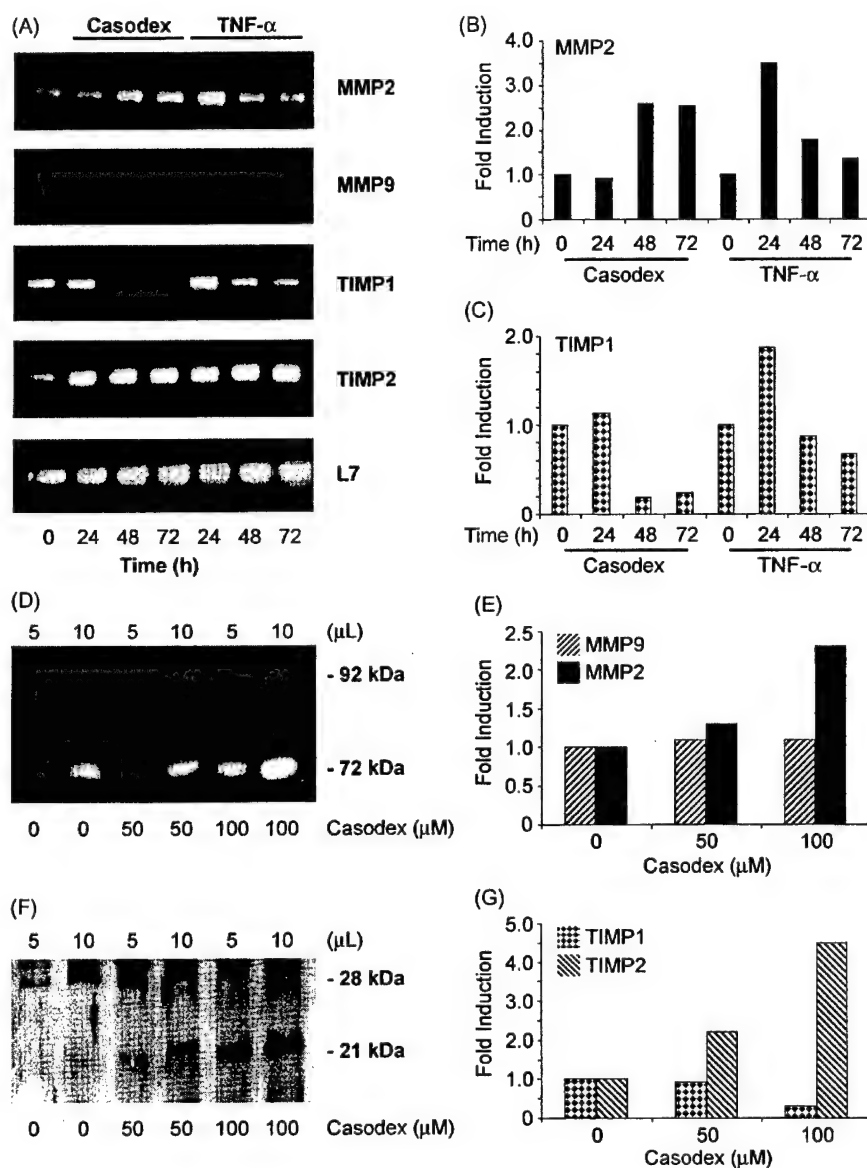


Fig. 6. RT-PCR analysis and zymography of genes associated with tumor invasion and metastasis after Casodex or TNF-α treatment. Steady-state mRNA levels of MMP2, MMP9, TIMP1, TIMP2 or L7 (an integral ribosomal protein on the large subunit) were measured by semi-quantitative RT-PCR from LNCaP cells after treatment with 10 μM Casodex or 10 ng/ml TNF-α for 0, 24, 48 or 72 h as described in section 2 (Panel A). Steady-state mRNA levels of MMP2 (Panel B) and TIMP2 (Panel C) were quantified by densitometric scanning. (Panel D) LNCaP cells were treated with 0, 50 or 100 μM Casodex in GC medium minus BSA for 72 h. After treatment, protease activity of the media was measured by zymography analysis as described in section 2. Level of protease activities was quantified by densitometric scanning (Panel E). Protease inhibitor activity of the media described above was measured by reverse zymography analysis as described in section 2 (Panel F) and level of protease inhibitor activities was quantified by densitometric scanning (Panel G).

72 h (Fig. 7, Panels A and C). Taken together, the mRNA expression level of several genes that have been implicated in tumor invasion and metastasis are altered after Casodex treatment, providing a mechanistic explanation for the acquisition of invasiveness in LNCaP cells.

4. Discussion

To test the hypothesis that hormone-dependent, non-metastatic, prostate cancer cells acquire an invasive phenotype after treatment with anti-androgens, we have established the

androgen-dependent, non-metastatic LNCaP prostate cancer cell line in serum-free, phenol red-free, chemically defined medium supplemented with 1 nM testosterone [21]. This culture system effectively recapitulates the low growth fraction seen in most prostatic tumors. Using this model system we have characterized the ability of Casodex, a relatively new second generation "pure" anti-androgen that retains its antagonistic properties in LNCaP cells despite the mutated androgen receptor present in these cells, to induce apoptosis. Time course analysis of this process, using flow cytometry to monitor changes in cell cycle kinetics, crystal violet to monitor changes in cell number, and TUNEL to monitor the

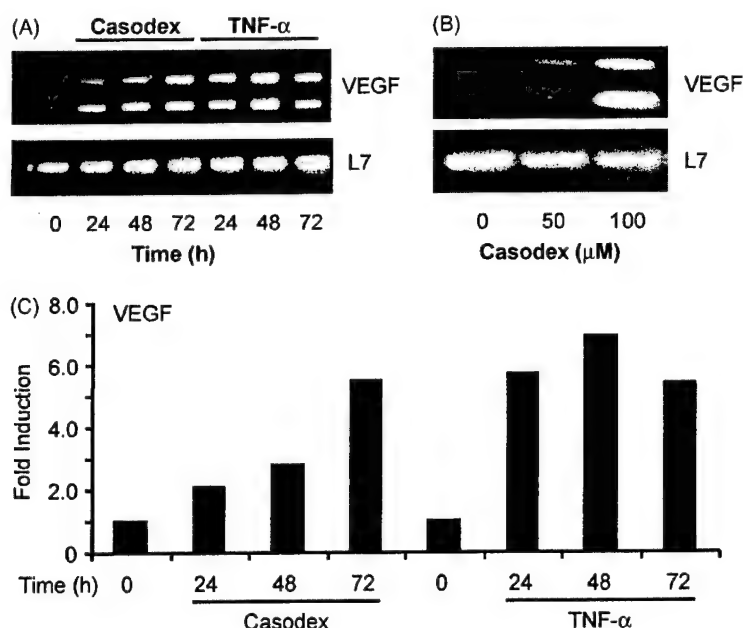


Fig. 7. RT-PCR analysis of VEGF after Casodex or TNF- α treatment. Steady-state mRNA levels of VEGF were measured by semi-quantitative RT-PCR from LNCaP cells after treatment with 10 μ M Casodex or 10 ng/l TNF- α for 0, 24, 48 or 72 h (Panel A) or 0, 50 or 100 μ M Casodex for 72 h (Panel B) as described in section 2. Steady-state mRNA levels of VEGF (all four isoforms) were quantified by densitometric scanning (Panel C).

apoptotic population, has demonstrated that both 10 ng/ml TNF- α and 50 μ M Casodex induce cell death in 40–50% of the LNCaP cells by 48 h and 65–80% by 72 h, regardless of whether testosterone is present in the medium or not. However, as demonstrated by agarose gel electrophoresis, DNA fragmentation in LNCaP cells after treatment with Casodex is considerably slower than TNF- α induced fragmentation and does not proceed beyond the 60 kb stage.

We have monitored the changes in the mRNA levels using RT-PCR and have shown that a number of ECM-proteases are induced by Casodex, while the steady-state mRNA levels of several of the protease inhibitors decrease during the time frame of these experiments. Using zymography, we have shown that although the 92 kDa gelatinase is not affected by Casodex, the 72 kDa gelatinase activity are markedly increased in LNCaP cells after treatment with Casodex, suggesting that there is a complex interaction between the ECM-proteases and their inhibitors, such that the treatment with either TNF- α or Casodex alters the balance in favor of protease activity.

To determine whether the surviving cells had acquired an invasive phenotype, surviving cells from each treatment were gently trypsinized and re-plated on 8 μ m membranes. The number of viable cells that migrated through the Matrigel-coated membrane was counted by phase contrast microscopy. Both microscopic inspection and additional MTT assays indicate that the parental LNCaP cells do not migrate through the membrane, either in the absence or presence of testosterone. However, cells that have been treated with Casodex show a dose-dependent, statistically significant, increase in the number of invasive cells, in the absence or presence of testosterone. We estimate that

0.2–0.4% of the surviving cells acquires the ability to invade through the membrane following anti-androgen treatment. While this represents a very small percentage of the surviving cells (and an even smaller proportion of the original cell number), these cells represent a clinically significant population of cells. It should be noted that a small 1.5 cm³ stage A1 or A2 tumor will contain in excess of 10⁹ cells while a 30 g stage B tumor may contain as many as 10¹¹ cells, suggesting that anywhere from 10⁶ to 10⁸ tumor cells may be rendered invasive, and potentially metastatic as a result of anti-androgen therapy.

These experiments raise several critical issues. Casodex induces a dose-dependent increase in apoptosis, and a dose-dependent increase in the number of invasive cells. This implies that a larger proportion of the surviving cells acquire the invasive phenotype at higher doses even though there are fewer surviving cells. Since neither the control, untreated cells nor the TNF- α -treated cells show the same acquisition of the invasive phenotype, it is very unlikely that this is merely the selection of a pre-existing sub-population of invasive cells. Secondly, since TNF- α induces as much or more apoptosis in LNCaP cells over the same time period, but does not induce an invasive phenotype, the mechanisms underlying the acquisition of the invasive phenotype are not common to all agents that induce apoptosis in these cells.

Many, if not all, of the ECM-proteases that are induced in dying cells are also expressed by metastatic tumor cells and have been associated with the invasive phenotype of these cells [22,23]. For example, MMPs and u-PA have been shown in vitro and in vivo to play an important role in tumor invasion and metastasis. The balance between MMPs and TIMPs has been shown to be altered in malignant

prostatic tissue samples compared to normal tissue [24]. Expression of u-PA in prostate cancer cell lines has been shown to correlate with metastatic potential, with the non-metastatic LNCaP cells expressing low levels, while the highly metastatic DU-145 cells express high levels of u-PA [25]. Thus, the proteases that are required for apoptotic elimination of superfluous or damaged cells are the same proteases that allow a metastatic cell to release itself from ECM so that it can invade another site. Other than DNA fragmentation, which completes the death process, dying and metastatic cells share an astounding number of similarities. This has lead us to hypothesize that the invasive phenotype observed in a small sub-set of cells treated with anti-androgens might in fact be the result of abrogated cell death. The delayed and incomplete fragmentation induced by Casodex in LNCaP cells may provide an opportunity for extensive, but inappropriate DNA repair, leading to genomic instability. The data presented in this manuscript provide evidence that anti-androgens (exemplified by Casodex) induce an invasive phenotype in prostate cancer cells that abrogate the apoptotic process.

These studies have significant implications. If the in vitro data presented here are verified in preclinical animal studies, and non-invasive androgen-dependent tumor cells show an increased propensity to metastatic progression in vivo after treatment with anti-androgens, it suggests that current monotherapies using anti-androgens for organ-confined prostate cancer are ill-advised. These data may also have significant implications for patients being treated in a neo-adjuvant setting with anti-androgens to debulk primary tumors prior to surgery, and with the use of anti-androgens in chemopreventive settings, since these treatments may lead to an increased risk of metastatic disease, and greater tumor burden later in life.

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APPENDIX TWO

Antiandrogen-induced cell death in LNCaP human prostate cancer cells

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Abstract

Antiandrogens such as Casodex (Bicalutamide) are designed to treat advanced stage prostate cancer by interfering with androgen receptor-mediated cell survival and by initiating cell death. Treatment of androgen sensitive, non-metastatic LNCaP human prostate cancer cells with 0–100 μ M Casodex or 0–10 ng/ml TNF- α induces cell death in 20–60% of the cells by 48 h in a dose-dependent manner. In cells treated with TNF- α , this is accompanied by the loss of mitochondrial membrane potential ($\Delta\Psi_m$) and cell adhesion. In contrast, cells treated with Casodex display loss of cell adhesion, but sustained mitochondrial dehydrogenase activity. Overexpression of Bcl-2 in LNCaP cells attenuates the induction of cell death by TNF- α but not Casodex, suggesting that mitochondria depolarization is not required for the induction of cell death by Casodex. While both TNF- α and Casodex-induced release of cytochrome *c* in LNCaP cell is predominantly associated with the translocation and cleavage of Bax, our data also suggest that Casodex induces cell death by acting on components downstream of decline of $\Delta\Psi_m$ and upstream of cytochrome *c* release. Furthermore, while induction of both caspase-3 and caspase-8 activities are observed in TNF- α and Casodex-treated cells, a novel cleavage product of procaspase-8 is seen in Casodex-treated cells. Taken together, these data support the hypothesis that Casodex induces cell death by a pathway that is independent of changes in $\Delta\Psi_m$ and Bcl-2 actions and results in an extended lag phase of cell survival that may promote the induction of an invasive phenotype after treatment.

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Keywords: casodex; prostate cancer; LNCaP; Bcl-2; Bax; cytochrome *c*; caspase; mitochondrial membrane potential

Abbreviations: AR, androgen receptor; AFC, 7-amino-4-trifluoromethyl coumarin; ANT, adenine nucleotide translocator; DISC, death-inducing signaling complex; $\Delta\Psi_m$, mitochondrial membrane potential; GAPDH, glyceraldehyde-3-phosphate de-

hydrogenase; IMM, inner mitochondrial membrane; MMP, matrix metalloprotease; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; OMM, outer mitochondrial membrane; PI, propidium iodine; PT, permeability transition; T₃, 3,3',5-triiodothyronine; t-Bax, truncated Bax; TMRE, tetramethylrhodamine ethyl ester; TNF- α , tumor necrosis factor- α ; VDAC, voltage-dependent anion channel.

Introduction

Active cell death, or apoptosis, plays a central role in maintaining tissue homeostasis and proper disposal of damaged or excess cells, including the epithelial cells of the prostate after castration or administration of antiandrogens.¹ Casodex, an antiandrogen used in prostate cancer therapy, is designed to reduce tumor size by interfering with normal androgen receptor (AR)-mediated processes that ensure prostate cell survival and by triggering tumor cells to undergo apoptosis.² However, tumors treated with antiandrogens ultimately become hormone refractory and have an increase propensity for metastasis.^{3–6}

Apoptosis is usually manifested by one of two major execution pathways downstream of death signals: the death receptor-mediated pathway, often referred to as the extrinsic pathway, and the mitochondrial pathway or intrinsic pathway.⁷ The death receptor-mediated pathway is activated upon ligand binding of cell surface death receptors such as tumor necrosis factor- α (TNF- α), initiating ligand-induced receptor trimerization and the formation of death-inducing signaling complex (DISC).^{8,9} Once caspase-8, the initiator caspase, is recruited in zymogen form to the DISC, it is autocatalytically processed and released from the complex to the cytosol as active tetramer to transactivate a number of downstream executioner caspases including the dominant executioner caspase, caspase-3.^{10–12} Caspase-3 in turn mediates the activation of a number of proteases and nucleases that are responsible for the loss of vital cell function, the condensation of the nucleus, and fragmentation of genomic DNA.^{13–15}

Like other death-promoting stimuli such as oxidative stress, calcium overload and ATP depletion, caspase-8 activation from the death-receptor pathway can also trigger the mitochondrial pathway that involves the release of cytochrome *c* from the intermembrane space to the cytosol, loss of mitochondrial membrane potential ($\Delta\Psi_m$), hyperdensity of the matrix, and shrinkage of the organelle.^{16–18} Once released from the mitochondria, cytochrome *c* assembles with Apaf-1 and procaspase-9 to form the apoptosome, which leads to the activation of caspase-3 and other executioner caspases that are responsible for the degradation of a variety of structural and housekeeping proteins, resulting in the disassembly of the cell.¹⁹

Key regulators of the mitochondrial pathway include both antiapoptotic and proapoptotic members of the Bcl-2 family of proteins. The antiapoptotic Bcl-2 subfamily includes Bcl-2 and Bcl-x_L, which have been shown to block the release of

cytochrome *c* and the decline in $\Delta\Psi_m$ by heterodimerizing with proapoptotic proteins and neutralizing their activities.⁷ The proapoptotic Bcl-2 subfamily includes Bax and Bak, are translocated from their cytoplasmic location to the mitochondria, where they induce the release of cytochrome *c* either through the formation of a multimeric channel or direct interaction with the voltage-dependent anion channel (VDAC) at the outer mitochondrial membrane (OMM).^{20,21} Cleavage of Bax to truncated t-Bax has also been shown to increase its cytotoxicity and has been implicated in facilitating the assemble of adenine nucleotide translocator (ANT) at the inner mitochondrial membrane (IMM) and VDAC at the OMM in the formation of the mitochondrial permeability transition (PT) pore complex, which is responsible for the dissipation of $\Delta\Psi_m$, matrix swelling, and the release of cytochrome *c*.²²⁻²⁴

To investigate the mechanism of Casodex-induced cell death in prostate cancer cells, we have compared specific intracellular events in the hormone-sensitive, nonmetastatic human prostate LNCaP cell line before and after treatment with Casodex and TNF- α . As Bcl-2 overexpression has been frequently observed in hormone refractory prostate tumors,²⁵ we have also examined the effect of Bcl-2 overexpression on the induction of cell death by Casodex. Unlike TNF- α , Casodex induces cell death through Bax-dependent and

-independent pathways that involve components downstream of decline of $\Delta\Psi_m$ and upstream of cytochrome *c* release.

Results

Sustained mitochondrial dehydrogenase activity and limited loss of $\Delta\Psi_m$ in Casodex-induced cell death

To investigate the mechanism of Casodex-induced cell death in LNCaP cells, we first compared specific morphological events in LNCaP cells after treatment with Casodex and TNF- α . TNF- α induces cell death in LNCaP cells in a time-dependent manner as measured by crystal violet assay which monitors changes in number of attached cells, and MTT which monitors changes in mitochondrial dehydrogenase activities. When LNCaP cells are treated with 5 ng/ml TNF- α for 24–72 h, 20–60% cell death is observed by both assays (Figure 1, panel A), suggesting that the loss of cell mitochondrial activity and cell attachment are coordinated events. When LNCaP cells are treated with 100 μ M Casodex, a similar time-dependent decrease in attached viable cells is seen for the first 24 h. However, after this initial decrease to 70% viability in mitochondrial dehydrogenase activity during the first 24 h,

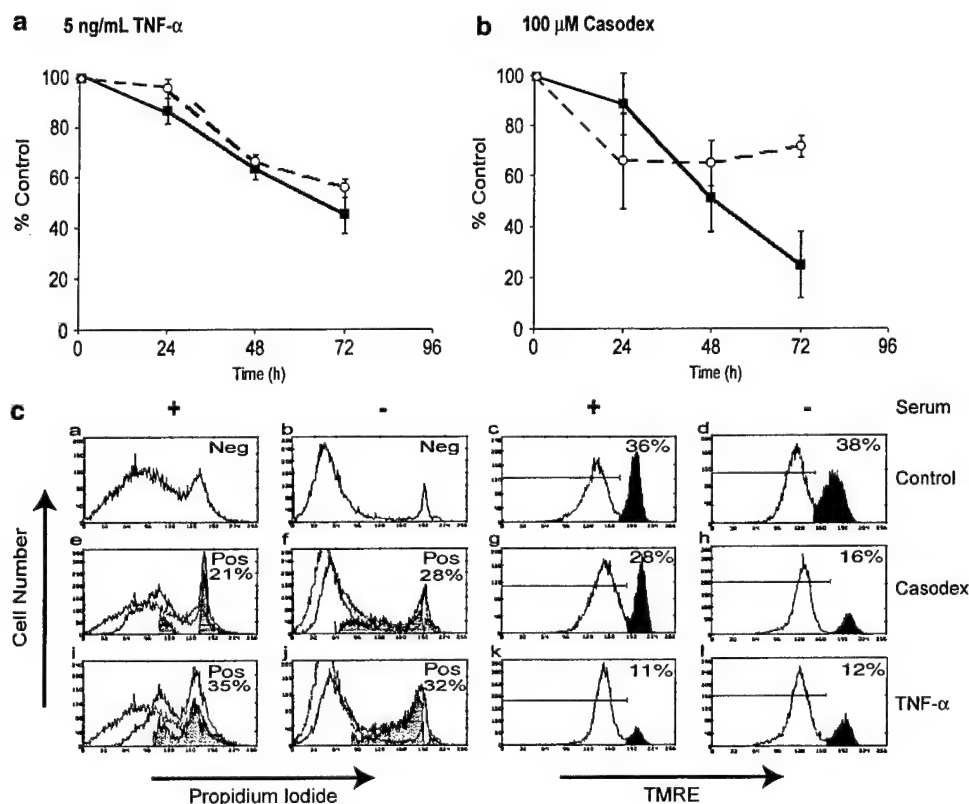


Figure 1 Effects of TNF- α and Casodex on cell adhesiveness, mitochondrial dehydrogenase activities and mitochondrial membrane potential ($\Delta\Psi_m$). LNCaP cells were treated with 5 ng/ml TNF- α (panel A) or 100 μ M Casodex (panel B) for 24, 48 and 72 h in Gc medium. Cell adhesiveness was determined by crystal violet (■) and mitochondrial dehydrogenase activity was measured using MTT assay (○). Results are expressed as mean \pm S.D. of three independent experiments. (panel C) LNCaP cells were treated with control vehicle (a–d), 50 μ M Casodex (e–h), or 5 ng/ml TNF- α (i–l) for 48 h in serum-containing medium (a, c, e, g, i, k) or serum free Gc medium (b, d, f, h, j, l). Cell death was measured by PI-exclusion (a, b, e, f, i, j). Shaded area represents the induction of cell death calculated by Multiplex AV software as lapped increase in PI staining due to cell membrane permeability following treatment. $\Delta\Psi_m$ was measured by TMRE-inclusion (c, d, g, h, k, l). Percentage of cells that maintains $\Delta\Psi_m$ was calculated by determining the integral of shaded area (which represents high TMRE staining) versus total area using Multiplus AV software

there is no further significant change in mitochondrial dehydrogenase activity (Figure 1, panel B). This suggests that Casodex and TNF- α induce cell death by mechanisms that are substantially different.

The loss of $\Delta\Psi_m$ is a critical intracellular event for apoptosis executed through the intrinsic mitochondrial pathway. As shown in Figure 1, panel C, compared to vehicle-treated control cells (Figure 1, panel C; a–d), TNF- α induces cell death and reduction of $\Delta\Psi_m$ in both serum-containing and serum-free medium (Figure 1, panel C; i–l). In contrast, Casodex only induces the reduction of $\Delta\Psi_m$ in serum-free medium (Figure 1, panel C; g, h), despite inducing similar amounts of cell death in both media (Figure 1, panel C; e, f). This suggests that Casodex is able to induce cell death without causing the dissipation of $\Delta\Psi_m$ if trophic factors are present in the serum to attenuate the process. This also suggests that, like the reduction of mitochondrial activities, the reduction of $\Delta\Psi_m$ is not required for Casodex-induced cell death.

Induction of viability in non-adherent cells treated with Casodex

One possible explanation for these observations is that when LNCaP cells are treated with Casodex, a significant number of cells detach from the cell culture monolayer without the concomitant loss of mitochondrial dehydrogenase activity and are thus, by definition, still viable (Figure 1, panel B). To verify this, anchorage-free cell viability of nonadherent LNCaP cells detached from the cell culture monolayer after treatment with control vehicle, 50 μ M Casodex or 5 ng/ml TNF- α was measured in a soft agar assay. As shown in Figure 2, Casodex induces at least a three-fold increase in the viability of nonadherent cells as compared to vehicle-treated cells. In contrast, when cells are treated with TNF- α , there is no evidence of viable nonadherent cells after treatments, demonstrating that while inducing cell death in the majority of the cell population, Casodex induces a small portion to detach from monolayer while maintaining viability.

Overexpression of Bcl-2 in LNCaP cells

Bcl-2 is thought to play an important role in both the intrinsic mitochondrial pathway and the progression of prostate cancer. To investigate the role of Bcl-2 in Casodex-induced cell death, we stably transfected LNCaP cells with human Bcl-2 and isolated two independent clones, LNCaP-B10 and LNCaP-B19 that overexpress Bcl-2. The majority of overexpressed Bcl-2 protein in these two clones is localized to NNMF, consistent with previous suggestions that Bcl-2 associates with mitochondria to prevent $\Delta\Psi_m$ disruption and cytochrome *c* release (Figure 3, panel A). Moreover, these transfected clones do not have altered level of expression of cytochrome *c* (data not shown), making these clones suitable models for studying the role of Bcl-2 in the attenuation of the intrinsic mitochondrial pathway in Casodex-induced cell death.

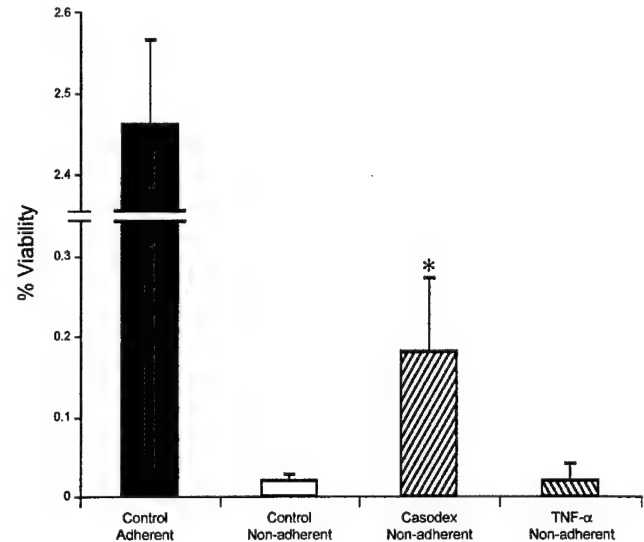


Figure 2 Viability of cells detached from the cell monolayer after treatment with TNF- α and Casodex. LNCaP cells were treated in triplicate with control vehicle, 50 μ M Casodex or 5 ng/ml TNF- α for 72 h in Gc medium. Viabilities of cells detached from the cell monolayer expressed as mean \pm S.D. were determined by anchorage-free cell viability assay. * P < 0.05; Casodex-treated versus control vehicle or TNF- α -treated as evaluated by analysis of variance

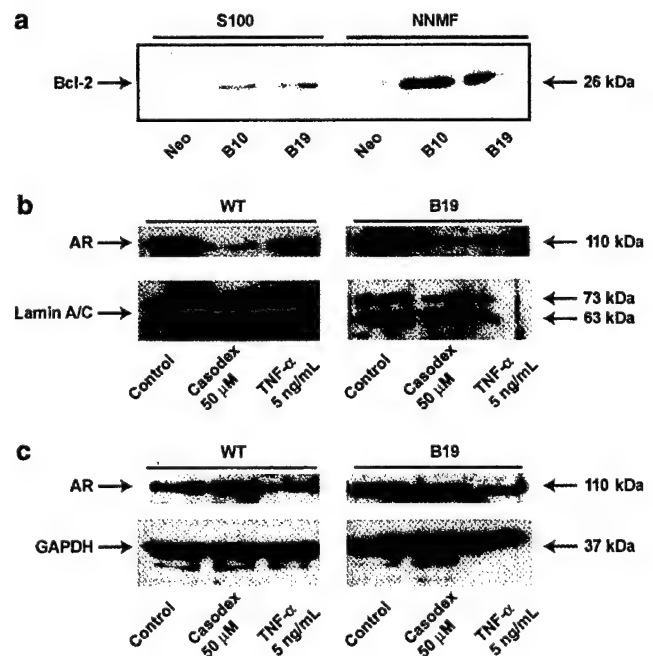


Figure 3 Nuclear translocation of androgen receptor (AR) in Bcl-2-overexpressing LNCaP cells after treatment with TNF- α and Casodex. (panel A) S100 and NNMF were isolated from empty vector transfected (Neo) and Bcl-2-overexpressing cells (B10 and B19), separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-Bcl-2 antibody (clone 124; Upstate Biotech.) as described in experimental procedures. (panels B and C) Nuclear fractions (panel B) and total cell lysate (panel C) were isolated from WT and Bcl-2 overexpressing (B19) LNCaP cells treated with control vehicle, 50 μ M Casodex or 5 ng/ml TNF- α for 48 h in Gc medium. Fractions (100 μ g) were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-AR antibody (PG-12, Upstate Biotech.) as described in experimental procedures

Nuclear localization but not expression of AR is altered by Casodex

To determine the subcellular localization and level of expression of AR after treatment with Casodex and TNF- α , total cell lysate and nuclear fractions of wild-type (WT) and Bcl-2-overexpressing (B19) LNCaP cells after treatment were prepared. Previous report has suggested that binding of Casodex to the mutated AR of LNCaP cells either restricts the nuclear translocation or induces the rapid degradation of the receptor.²⁶ AR is barely detectable in the nuclear fraction after treatment with Casodex (Figure 3, panel B). Untreated WT and Bcl-2 overexpressing (B19) LNCaP cells express substantial levels of AR in the nuclear fraction, both before and after treatment with 5 ng/ml TNF- α . In contrast, total level of expression of AR in both WT and Bcl-2 overexpressing (B19) LNCaP cells is not affected by Casodex treatment (Figure 3, panel C). In contrast, TNF- α treatment of both WT and B19 LNCaP cells only slightly decreases the level of AR, suggesting that Casodex acts on the androgen receptor-mediated pathway through reducing the nuclear localization of AR but not its expression.

Bcl-2 overexpression attenuates cell death induced by TNF- α but not Casodex

As shown in Figure 4, TNF- α induces the formation of apoptotic bodies only in WT (LNCaP-WT) but not in Bcl-2 overexpressing (LNCaP-B10) LNCaP cells (Figure 4, panel A; c, f). In contrast, Casodex induces the formation of apoptotic bodies in both WT (LNCaP-WT) and Bcl-2 overexpressing (LNCaP-B10) LNCaP cells (Figure 4, panel A; b, e). This suggested that Bcl-2-overexpression, which inhibits the release of cytochrome *c* and the dissipation of $\Delta\Psi_m$, rescues LNCaP-B10 cells from TNF- α but not Casodex-induced cell death. To verify this, cell viability of LNCaP-WT, LNCaP-B10, LNCaP-B19 and LNCaP-Neo cells after treatment with control vehicle, Casodex or TNF- α was measured. While overexpression of Bcl-2 in LNCaP cells attenuates TNF- α -induced reduction in cell viability (Figure 4, panel C), Casodex-induced reduction in cell viability is not altered by Bcl-2 overexpression (Figure 4, panel B). This demonstrates that Casodex induces cell death in the B10 and B19 cell lines, despite the elevated level of Bcl-2, and suggests that Casodex induces cell death by acting on components of the death pathway downstream of Bcl-2.

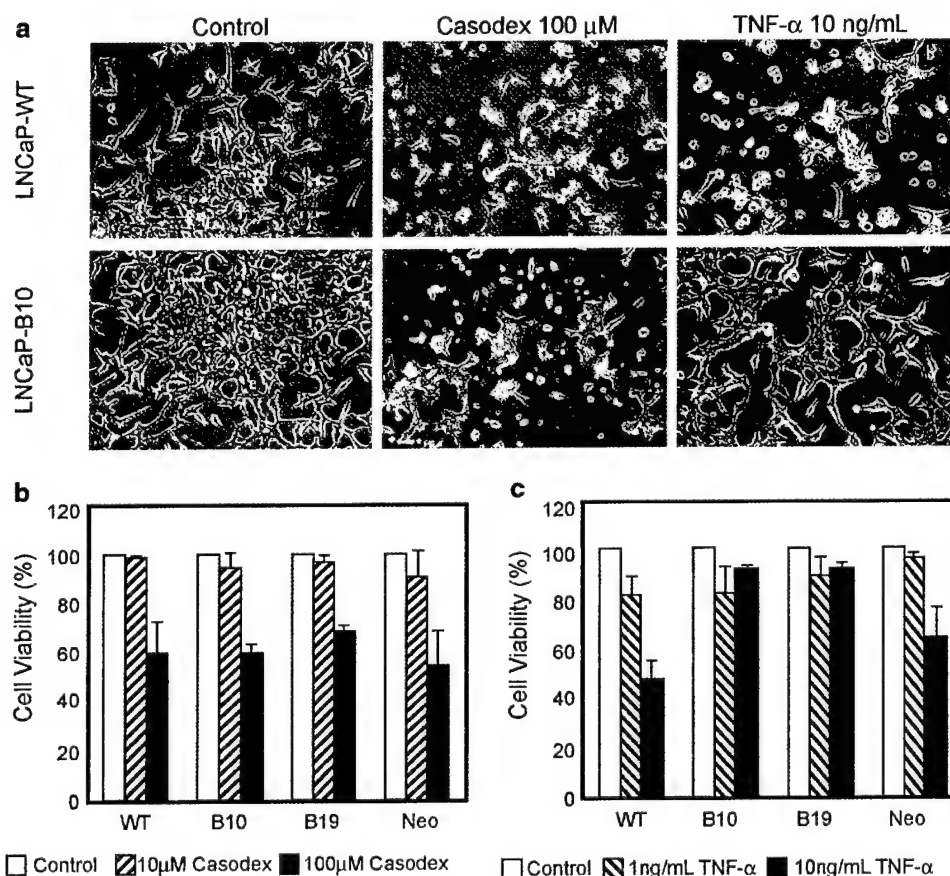


Figure 4 Effects of Casodex and TNF- α on cell viability of LNCaP cells overexpressing Bcl-2. (panel A) Phase contrast photograph of WT (LNCaP-WT; a, b, c) or Bcl-2-overexpressing (LNCaP-B10; d, e, f) LNCaP cells treated with control vehicle (a, d), 100 μ M Casodex (b, e) or 10 ng/ml TNF- α (c, f) for 72 h in Gc medium (Bar: 200 μ m). WT, Bcl-2-overexpressing (B10 and B19), and empty vector-transfected (Neo) LNCaP cells were treated with 10 or 100 μ M Casodex (panel B) or 1 or 10 ng/ml TNF- α (panel C), for 72 h in Gc medium. Cell viabilities from three independent experiments expressed as mean \pm S.D. were determined by MTT assay (Sigma)

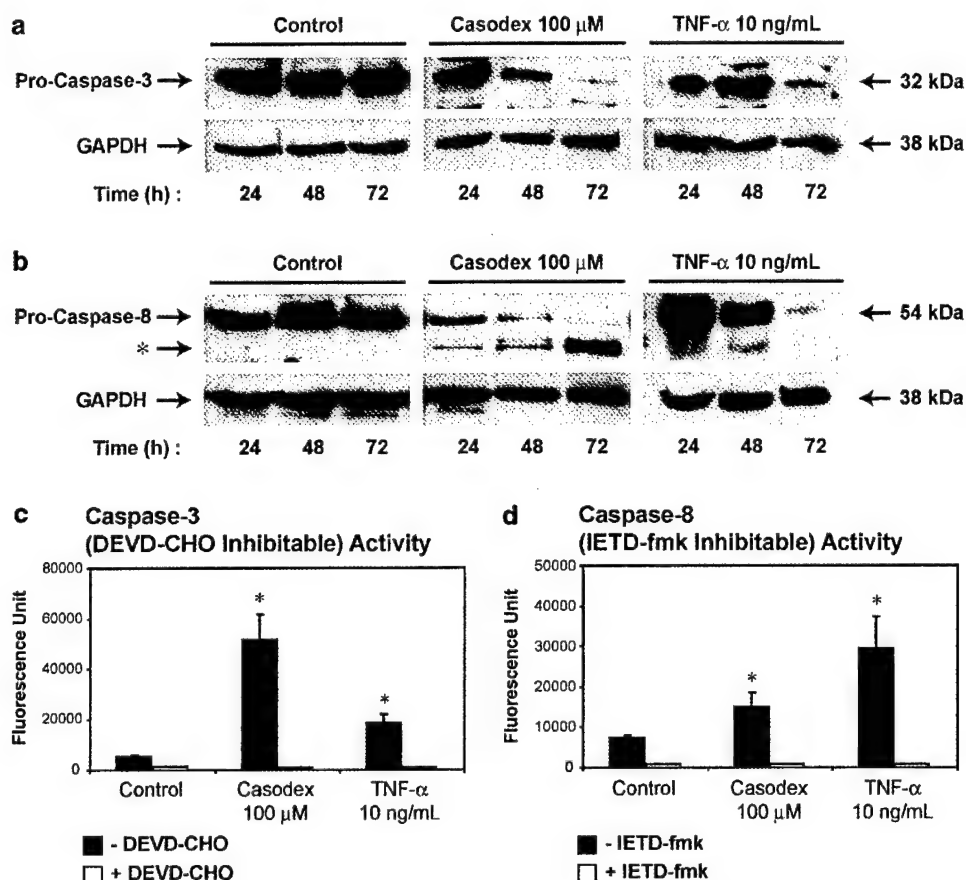


Figure 5 Activation of caspase-3 and caspase-8 after treatment with TNF- α and Casodex. Total cell lysate from WT LNCaP cells treated with control vehicle, 10 ng/ml TNF- α or 100 μ M Casodex for 24, 48 and 72 h in Gc medium were separated on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anticaspase-3 (06735, Upstate Biotech.) (panel A) or anticaspase-8 (clone 5F7, Upstate Biotech.) antibody (panel B). Cytosolic extracts of WT LNCaP cells (1×10^6 cells) treated with control vehicle, 10 ng/ml TNF- α or 100 μ M Casodex for 48 h in Gc medium were incubated with DEVD-AFC (panel C) or IETD-AFC (panel D) for 1 h at 37°C and analyzed by fluorescence spectrophotometry. DEVDase activities and IETDase activities represented as mean \pm s.d. were determined by ApoAlert Caspase Fluorescent Assay. * $P < 0.05$; Casodex or TNF- α -treated versus control vehicle-treated as evaluated by analysis of variance

Differential activation of caspases by Casodex and TNF- α

Caspase-3 and caspase-8 are proteolytically cleaved and activated directly downstream of the mitochondrial pathway or the death receptor pathway, respectively.^{27,28} As shown in Figure 5, panel A, procaspase-3 is proteolytically cleaved and activated after treatment with 100 μ M Casodex or 10 ng/ml TNF- α . In particular, the activation of caspase-3 in LNCaP cells by Casodex is observed 24–48 h after treatment, preceding that seen after TNF- α treatment where the activation does not occur until 48–72 h. Proteolytic cleavage of procaspase-8 occurs between 48 and 72 h after treatment with Casodex or TNF- α . However, Casodex treatment induces a novel 45 kDa cleavage product of procaspase-8 (Figure 5, panel B). To determine whether this cleavage product is enzymatically active, both caspase-3 and caspase-8 activities of LNCaP cells were measured using fluorogenic substrate DEVD-AFC and IETD-AFC respectively, before and after treatment with TNF- α or Casodex. Casodex induces a higher DEVDase activity than TNF- α in LNCaP cells after 48 h of treatment (Figure 5, panel C), agreeing with the Western

blot analysis (Figure 5, panel A). Furthermore, even though Casodex induces unusual cleavage of procaspase-8, the cleavage product retains IETDase activity, although at a reduced level compared to TNF- α (Figure 5, panel D).

Casodex-induced release of cytochrome C involves both Bax-dependent and -independent pathways

The release of cytochrome *c* from mitochondria to cytosol is one of the hallmarks for apoptosis. Since the translocation of Bax from the cytosolic to the mitochondrial fraction is often associated with the release of cytochrome *c* during apoptosis,²⁹ we examined the subcellular redistribution of cytochrome *c* and Bax after Casodex treatment of LNCaP cells. S100 and NMF were isolated from WT LNCaP cells after treatment with Casodex or TNF- α . ATP synthase- α was not detectable in S100, demonstrating that the S100 cytosolic fraction is free of mitochondrial contamination (Figure 6, panels A and B). Increased levels of cytochrome *c* are detected in S100 after Casodex or TNF- α treatment (Figure 6,

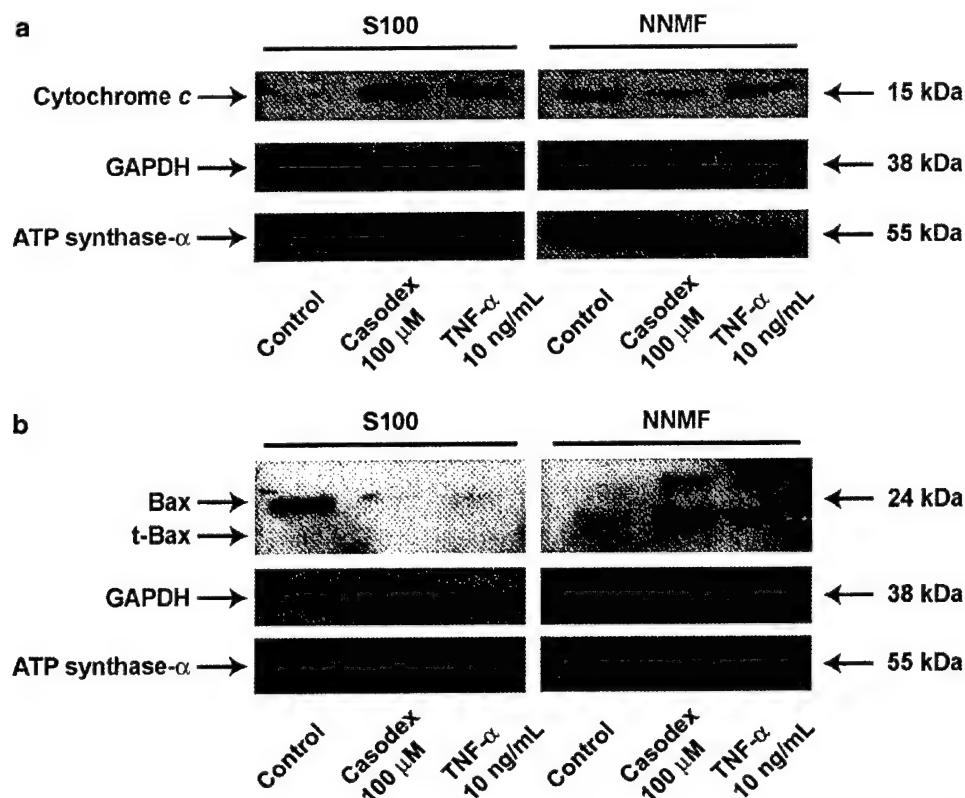


Figure 6 Translocation of cytochrome *c* and Bax after treatment with TNF- α and Casodex. S100 and NNMF isolated from WT LNCaP cells treated with control vehicle, 10 ng/ml TNF- α or 100 μ M Casodex for 48 h in Gc medium were separated on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anticytochrome *c* (7H8.2C12, PharMingen) (panel A) or anti-Bax (13666E; PharMingen) (panel B) antibody. Anti-ATP synthase- α (A11177; Molecular Probes) and anti-GAPDH (6G5; Biogenesis) antibodies were used to immunoblot for the level of mitochondrial and cytoplasmic contamination respectively

panel A), suggesting that cytochrome *c* is relocated from the mitochondria to the cytosol upon treatment. Furthermore, decreased levels of Bax in S100 and increased level of both Bax and truncated Bax (t-Bax) in NNMF occur after treatment with both Casodex and TNF- α (Figure 6, panel B), demonstrating that in LNCaP cells, both Casodex and TNF- α induce the cleavage of Bax and its localization to the mitochondria. Further examination by immunofluorescence microscopy suggested that Casodex-induced release of cytochrome *c* is executed predominately by a Bax-dependent mechanism. As shown in Figure 7, in control vehicle-treated cells (Figure 7; a–d), punctated cytochrome *c* staining is observed and Bax-staining is undetectable, consistent with their respective mitochondrial and cytoplasmic location in preapoptotic cells. Upon induction of apoptotic signal by TNF- α (Figure 7; e–h) or Casodex (Figure 7; i–l; m–p), diffuse cytoplasmic cytochrome *c* staining is detected throughout the cell, which obscure the nuclei, consistent with redistribution of cytochrome *c* from mitochondria to cytoplasm (Figure 7; g, k, o). This is accompanied by translocation of Bax from cytoplasm to mitochondria as evidenced by punctuated Bax staining in cells treated with TNF- α or Casodex (Figure 7; f, j, n). This is followed by chromatin condensation as identified by Hoechst nuclear staining and cytosolic vacuolization and nuclear condensation (Figure 7; e, i, m). The presence of cytochrome *c* release in the apparent absence of Bax translocation is seen

in a small proportion (<20%) of cells treated with casodex but not TNF- α (Figure 7; l–p, inset), leaving open the possibility that some cells may release cytochrome *c* in the absence of Bax translocation.

Discussion

Prostate cancer is the second leading cause of cancer-related deaths of men in Western countries. Since most prostatic tumors are androgen dependent, androgen deprivation through surgical or medical castration remains the most common treatment despite their considerable side effects on sexual potency. Monotherapy with antiandrogens such as Casodex, which may preserve testosterone levels and sexual potency, has provided an attractive alternative therapeutic approach to surgical intervention.³⁰ Unfortunately, progression to hormone refractory diseases occurs within a few years in nearly all cases. Although the underlying mechanism of Casodex-induced prostate cancer cell death has yet to be fully elucidated, the use of Casodex alone, or as adjuvant to treatment, has remain to be an attractive alternative to castration, mainly due to its quality-of-life and tolerability benefits.

Unlike TNF- α , Casodex induces the loss of cell adhesion in LNCaP cells prior to the loss of mitochondrial activity. In

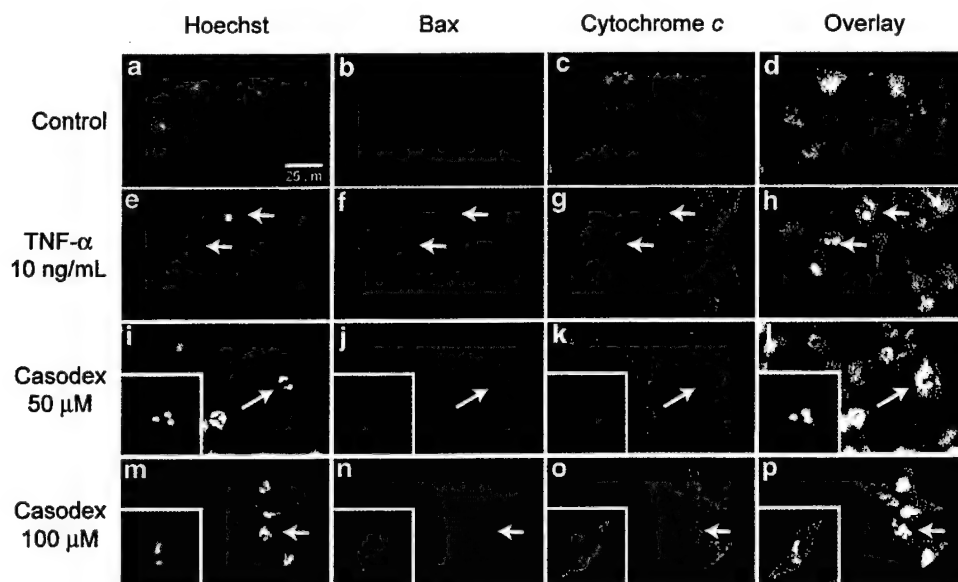


Figure 7 Effects of TNF- α and Casodex on cytochrome *c* release, Bax translocation, and nuclear condensation. LNCaP cells were treated with control vehicle (a–d), 10 ng/ml TNF- α (e–h), 50 μ M (i–l) or 100 μ M Casodex (m–p) for 48 h in Gc medium, fixed and immunostained with anticytochrome *c* mouse monoclonal (6H2.B4, PharMingen) and anti-Bax rabbit polyclonal (06499MN; Upstate Biotech.) antibody, and visualized with anti-mouse ALEXA-488-conjugated and anti-rabbit ALEXA-568-conjugated (A11001, A11036; Molecular Probes) secondary antibody respectively. Nuclei were counterstained with Hoechst 33258 (Sigma) (Bar: 25 μ m)

addition, the observation that Casodex is able to induce cell death without disrupting $\Delta\Psi_m$ further demonstrates that mitochondria are unlikely to be the immediate target of Casodex-induced cell death. This suggests that after treatment with Casodex, some nonadherent cells are viable. This is further supported by the demonstration that after Casodex treatment, a small proportion of the nonadherent cells are not only viable immediately after treatment, but remain so for several weeks and are capable of cell division and colony formation. Furthermore, extracellular matrix proteases including MMP-2 and MMP-9 are upregulated in Casodex-treated LNCaP cells.³¹ Taken together, these data suggest that while Casodex induces cell death in most LNCaP cells, a small proportion of cells do not lose their mitochondrial membrane potential or mitochondrial dehydrogenase activity and upregulate several extracellular matrix proteases, rendering them capable of invasion.

The invasive phenotype is a critical step in the progression of prostate cancer. The majority of prostate cancer patients treated with Casodex or other antiandrogens ultimately develop hormone refractory diseases and have increased propensity for metastasis. While the mechanism of Casodex-induced cell death in prostate cancer cell is presumed to be through the AR (since it is blocked by pre-incubation with an androgen analogue R1881)⁴⁶ it is not clear how binding to AR induces apoptotic responses. Since abrogation of cell death in cancer cells has been suggested to lead to cancer progression,³² the underlying mechanism of Casodex-induced cell death needed to be fully elucidated. It has been suggested that failure of conventional androgen deprivation therapy in prostate cancer may be caused by clonal expansion of tumor cells that are able to continue androgen-dependent growth despite of low concentrations of serum androgens through the amplification or mutation of the AR gene.³³ While Casodex downregulates AR in the nucleus through cytoplasmic

translocation and degradation, neither Bcl-2 overexpression nor TNF- α alter the expression or nuclear localization of AR. This suggests that neither TNF- α nor Casodex induce cell death simply by affecting the levels of AR expression. Furthermore, the fact that Bcl-2 overexpression attenuates cell death induced by TNF- α but not Casodex suggested that increased Bcl-2 expression seen in advance stage prostate tumor²⁵ is not necessary the cause of survival for hormone refractory cancer cells after antiandrogen therapy. Further studies are needed to elucidate the role of Bcl-2 overexpression in the resistance to antiandrogen therapy.

A central component of the apoptotic machinery is a proteolytic system involving a family of proteases called caspases where activation through irreversible proteolysis of their inactive precursors (zymogens) at specific Asp residues is required.²⁸ While TNF- α initiates this proteolytic system through caspase-8, Casodex appears to induce it primarily through caspase-3. Despite the uncertainties around the precise sequence of cleavage of procaspase-8, Casodex appears to induce a nonclassical cleavage product of caspase-8 that may be due to caspase-3 cleavage of procaspase-8 at D³⁹⁵EAD↓F³⁹⁹, resulting in the novel cleavage product at 45 kDa.³⁴ Although caspase-8 is moderately active after Casodex treatment, it is nevertheless generated from an alternative pathway where substrate specificity of the active enzyme may be altered. This may in turn change the temporal sequence of events surrounding the activation of caspase-8 and the remainder of the apoptotic cascade and providing an opportunity for cells to avoid DNA cleavage and cell death.

The translocation and cleavage of Bax is often associated with mitochondrial disruption during apoptosis.²⁹ We have demonstrated that the release of cytochrome *c* after Casodex treatment is predominately associated with the translocation and cleavage of Bax in a $\Delta\Psi_m$ -independent manner. In-creas-

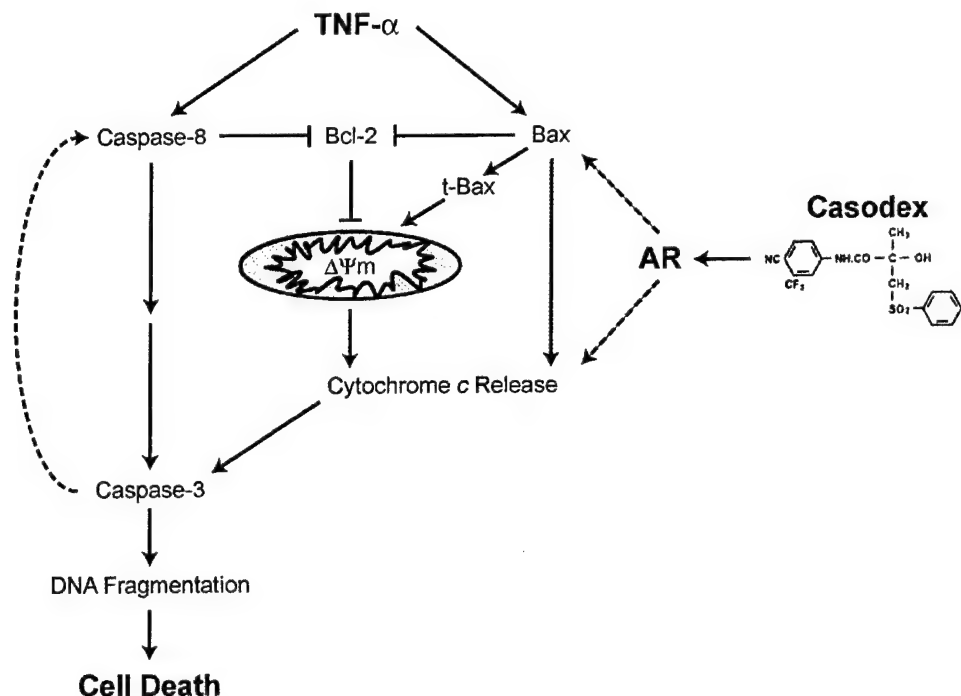


Figure 8 Schematic model of proposed mechanism of Casodex-induced cell death. Upon ligand binding of cell surface death receptors such as TNF- α , the initiator caspase, procaspase-8 is recruited and auto-catalytically activated. Bax is also activated by cleavage to t-Bax and binds to the mitochondrial membrane, inducing the reduction of $\Delta\Psi_m$ and the release of cytochrome *c*. The activation of caspase-8 and release of cytochrome *c* into the cytoplasm results in the activation of caspase-3, an executioner caspase, which activates the nucleases and proteases responsible for nuclear condensation and subsequent DNA fragmentation, leading to cell death. These processes can be blocked by overexpression of Bcl-2. In contrast, Casodex induces the release of cytochrome *c* by both Bax-dependent and -independent mechanisms (dotted arrows), resulting in the activation of caspase-3, DNA fragmentation and cell death that is independent of changes in $\Delta\Psi_m$ and insensitive to the overexpression of anti-apoptotic Bcl-2 protein. Caspase-8 is catalytically activated by Casodex through a mechanism that is mediated by caspase-3 or other executioner caspases (dotted arrows)

ing evidence has suggested that the release of cytochrome *c* and disruption of $\Delta\Psi_m$ are separate events in the processes leading to mitochondrial disruption.^{35–37} Whether or not mitochondria plays an essential role in the control of apoptosis is still a matter of debate. In fact, our data suggest that mitochondrial membrane transition may serve as an amplifying loop for the apoptotic process at the later stages of apoptosis but may not play a direct role in the initiation of Casodex-induced cell death. These findings lead to the hypothesis that Casodex induces cell death by acting on components downstream of the decline in $\Delta\Psi_m$ and upstream of cytochrome *c* release in a predominantly Bax-dependent manner (Figure 8). There is also some evidence of Bax-independent release of cytochrome *c* after treatment with Casodex (Figure 8, inset). Whether this represents an alternative apoptotic pathway remains to be elucidated, however, there is precedent for Bax-independent cytochrome *c* release in neural cells, isolated liver mitochondria and HL-60 cells.^{36,38–41}

In summary, we have demonstrated that different drugs may induce cell death in the same cell line through different mechanisms that involve many or all of the same components of the apoptotic machinery, but with substantially different time courses and efficiencies. In particular, Casodex induces cell death in a lentigrade fashion that results in an extended lag phase of cell survival between the initiation of cell death and the fragmentation of DNA, during which time other

survivals mechanisms may abrogate the process. This may offer the mechanistic explanation for the failure of most antiandrogen therapy in prostate cancer and the emergence of hormone refractory tumors that have high propensity for metastasis, and raises questions about the use of Casodex and other antiandrogens for neoadjuvant therapy or as chemopreventive agents.

Materials and Methods

Cell culture

LNCaP cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS). Prior to each experiment, cells were plated in this medium, and after 24 h transferred to serum-free growth control (Gc) medium (RPMI-1640, 2 mg/ml BSA-V, 1 ng/ml EGF, 0.5 mg/ml fetuin, 50 nM hydrocortizone, 20 μ g/ml insulin, 25 nM sodium selenite, 0.5 mM sodium pyruvate, 0.1 nM T_3 , 10 μ g/ml transferrin, 10 nM testosterone)⁴² for an additional 48 h prior to treatment. This medium induces cell cycle arrest in LNCaP cells, lowering the fraction of cells in S phase to less than 10%, mimicking the growth fraction seen in most prostate tumors *in vivo*.⁴² Cell adhesiveness was assayed by crystal violet and mitochondrial dehydrogenase activity was measured by MTT assay (Sigma, St. Louis, MO, USA).

Cells were treated with 1–10 ng/ml TNF- α or 10–100 μ M Casodex dissolved in 95% ethanol and 5% dimethyl sulfoxide (DMSO). The doses of Casodex used in these experiments (10–100 μ M) were chosen

because they effectively bracket the steady-state serum concentrations ($C_{ss}=8.9 \pm 3.5 \mu\text{g/ml}$ [$30 \mu\text{M}$]), achieved in clinical studies with localized prostate cancer in which patients received 50 mg Casodex once per day,⁴³ either alone or in combination with Zoladex (an LH-RH analog). Casodex is also being used in a monotherapy regimen as an alternative treatment to castration in patients with locally advanced prostate cancer at a dose of 150 mg Casodex/day ($C_{ss} \approx 90 \mu\text{M}$).⁴⁴ Thus the upper dose used in our studies ($100 \mu\text{M}$) is only slightly higher than the steady-state serum levels achieved in the preclinical analysis of the pharmacokinetics of Casodex.

Flow cytometry

For analysis of $\Delta\Psi_m$, cells treated with control vehicle, 50 μM Casodex or 5 ng/ml TNF- α in serum-containing or serum-free medium for 48 h were harvested by trypsinization, resuspended in serum-containing medium with 1 mM tetramethylrhodamine ethyl ester (TMRE, Molecular Probes, Eugene, OR, USA), incubated for 15 min at 37°C, and analyzed for red fluorescence on FL3 using a 620 nm band-pass filter on an Epics XL Flow Cytometer. The results were modeled with the Multiplus AV software (Phoenix Flow Systems, San Diego, CA, USA).

For analysis of cell death, adherent cells were harvested by trypsinization, pooled with nonadherent cells and resuspended in serum-containing medium with 1 $\mu\text{g/ml}$ propidium iodide (PI). After incubation for 30 min at room temperature, samples were analyzed and modeled for PI fluorescence as described above.

Anchorage-free cell viability assay

LNCaP cells were treated with control vehicle, 50 μM Casodex or 5 ng/ml TNF- α in Gc medium for 72 h. After each treatment, nonadherent cells (5×10^3) detached from the cell culture monolayer were collected and replated on 6-well tissue culture plates (9.5 cm² of growth area per well; Corning Inc., Corning, NY, USA) in RPMI-1640 supplemented with 10% FBS and 0.5% DNA grade agarose (Life Technologies, Rockville, MD, USA). Cells were incubated at 37°C for a further 2 weeks, at which time the number of visible colonies per well was scored as a percentage of the number of cells replated.

Stable transfection

LNCaP cells were stably transfected with human Bcl-2 subcloned into pcDNA3 (Invitrogen, Carlsbad, CA, USA) or the empty vector using Lipofectin (Life Technologies). Transfected cells were selected with 400 $\mu\text{g/ml}$ G418 for 4 weeks. Independent clones were selected by serial dilution and two lines overexpressing Bcl-2 were designated LNCaP-B10 and LNCaP-B19, respectively. Cell lines demonstrating G418 resistance and transfected with the empty vector were designated LNCaP-Neo control cells.

Total cell lysate

Total cell lysates were prepared using standard protocols. Cells were trypsinized, and pelleted by centrifugation at $1500 \times g$ for 3 min at 4°C. Pellets were resuspended in Buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM HCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 250 mM sucrose, 10 mM sodium fluoride, 10 mM sodium vanadate, 10 $\mu\text{g/ml}$ Leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 $\mu\text{g/ml}$ Aprotinin and 1 mM benzamide), sonicated three times for 20 s, and stored at -80°C. Protein concentrations were determined by the Micro BCA protein assay (Pierce, Rockford, IL, USA).

Subcellular fractionation

Subcellular fractions were isolated as previously described.⁴⁵ Cells were trypsinized, and pelleted by centrifugation at $1500 \times g$ for 3 min at 4°C. Pellets were resuspended with three volumes of Buffer A and lysed with a Dounce homogenizer. Homogenates were centrifuged twice at $2000 \times g$ for 5 min at 4°C and the nuclear pellets were resuspended in Buffer A, sonicated for 20 s, and stored at -80°C. The supernatants were ultracentrifuged at $100\,000 \times g$ for 1 h at 4°C. The resultant pellets containing the mitochondria, and designated the non-nuclear membrane fractions (NNMF) were resuspended in Buffer A, sonicated for 20 s, and stored at -80°C. The resulting supernatant containing cytosolic fraction was designated S100 and stored at -80°C.

Western blot analysis

Total cell lysate (100 μg) and subcellular fractions (100 μg) isolated as described above were solubilized in loading buffer containing 2.5% β -mercaptoethanol, separated by SDS-PAGE, and transferred to nitrocellulose. Equal loading and transfer of proteins were confirmed by Ponceau-S staining (BDH, Dorset, England). Protein derived from total cell lysate, nuclear, NNMF and/or S100 extracts were immunoblotted with anti-AR rabbit polyclonal (PG-21; Upstate Biotechnology, Lake Placid, NY, USA), anti-Bcl-2 mouse monoclonal (clone 124; Upstate Biotech.), anti-Bax rabbit polyclonal (13666E; PharMingen, San Diego, CA, USA), anticaspase-3 rabbit polyclonal (06735; Upstate Biotech.), anticaspase-8 mouse monoclonal (clone 5F7; Upstate Biotech.), anticytochrome c mouse monoclonal (7H8.2C12; PharMingen), anti-GAPDH mouse monoclonal (6G5; Biogenesis, Kingston, NH, USA), antilamin A/C goat polyclonal (N-18; Santa Cruz Biotech., Santa Cruz, CA, USA) or anti-ATP synthase- α mouse polyclonal antibodies (A11177; Molecular Probes) diluted in blocking solution (1% heat denatured casein in PBS). Specific antibody binding was detected by goat anti-mouse (Caltag, Burlingame, CA, USA), goat anti-rabbit (BIORAD, Hercules, CA, USA), or mouse anti-goat (Jackson Immuno., West Grove, PA, USA) IgG antibody conjugated with horseradish peroxidase diluted 1:4000 or 1:8000 in blocking solution and autoradiographed with enhanced chemiluminescence (Pierce). Blots were stripped with Western Re-Probe™ Buffer (Geno Technology, St. Louis, MO, USA).

Caspase activity assay

Caspase activity was analyzed with the ApoAlert Caspase Fluorescent Assay kit according to manufacturer's protocol (CLONTECH, Palo Alto, CA, USA). In brief, cytosolic extracts from 1×10^6 LNCaP cells after treatment with control vehicle, 100 μM Casodex or 10 ng/ml TNF- α for 48 h in Gc medium were incubated with 50 μM DEVD-AFC or 50 μM IETD-AFC for 1 h at 37°C in the presence or absence of 10 μM DEVD-CHO or 10 μM IETD-fmk. Caspase-3 and caspase-8 activities of the samples were assayed by fluorescence spectroscopy with excitation at 390 nm and emission at 510 nm, of 7-amino-4-trifluoromethyl coumarin (AFC) after cleavage from the peptide substrate DEVD-AFC and IETD-AFC, respectively.

Immunofluorescence

LNCaP cells grown on Lab-Tek II chamberslides (Nalge Nunc International) were treated with control vehicle, 50 or 100 μM Casodex or 10 ng/ml TNF- α in Gc medium for 48 h. The cells were fixed in 3.7% formaldehyde in PBS for 5 min at room temperature, permeabilized in

methanol at -20°C for 6 min, and blocked overnight with 1% BSA in PBS containing 0.02% sodium azide. The slides were then incubated with anti-cytochrome *c* mouse monoclonal (6H2.B4; PharMingen) and anti-Bax rabbit polyclonal (06499MN; Upstate Biotech.) antibody, both diluted 1:100 in blocking buffer, for 2 h at 37°C in a humidified chamber. Slides were washed three times for 5 min with PBS followed by incubation for 1 h at room temperature with anti-mouse ALEXA-488-conjugated and anti-rabbit ALEXA-568-conjugated secondary antibody (photo-stable dyes with spectral properties similar to fluorescein; A11001, A11036; Molecular Probes) diluted 1:50 and 1:100 in blocking buffer, respectively. Slides were washed three times for 5 min with PBS, incubated for 15 min at room temperature with $1\text{ }\mu\text{g/ml}$ Hoechst 33258 (Sigma), washed five times for 5 min with PBS, rinsed with ddH_2O , and coverslipped with an antifade-mounting medium (Biomed, Foster City, CA, USA). Fluorescence was detected using Olympus AX70 microscope equipped with a Spot RT digital camera. Images were analyzed and merged using Adobe Photoshop software (Adobe Systems Inc., San Jose, CA, USA).

Statistical analysis

Data are expressed as means \pm S.D., and significance was determined by using a Student's *t*-test. A value of $P < 0.05$ was considered to be significant.

Acknowledgements

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APPENDIX THREE

PROGRAMMED CELL DEATH AND SURVIVAL PATHWAYS IN PROSTATE CANCER CELLS

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Programmed cell death, or apoptosis, is a series of morphologically and biochemically related processes. The extrinsic (death receptor mediated) and intrinsic (mitochondrial-mediated) apoptotic pathways can be triggered by physiological and pharmacological substances. However, other molecular events influence the sensitivity of prostate cancer cells to apoptotic stimuli, leading to marked variations in the responsiveness of prostate cancer cell lines to individual stimuli. Modulation of apoptotic responses by over expression of anti-apoptotic proteins (NF- κ B, IAPs and Bcl-2), or attenuation of pro-apoptotic proteins (PTEN and Bax) may be responsible for the variations in sensitivity of these cell lines to hormone and chemotherapy. The expression of anti- and pro-apoptotic proteins in some of the widely used *in vitro* models of prostate cancer is reviewed.

Q1 Keywords ■

INTRODUCTION

Most therapeutics for prostate cancer activate the apoptotic machinery in prostate cancer cells. Regardless of the initiating event, epithelial cells from the normal prostate and most tumor cells undergo similar morphological processes, although the timing of the events may vary from cell to cell (Fig. 1). Apoptosis in the prostate is initiated by either extrinsic pathways or intrinsic pathways or a combination of these pathways (Fig. 2).

In addition to the appearance of apoptotic bodies, there are several key apoptotic events that can be used to monitor programmed cell death. This includes DNA Fragmentation, Annexin V staining and caspase activation. Assessment of apoptosis using these endpoints, however, need to be used with some caution.

1. **DNA Fragmentation:** Most prostate cancer cells have the enzymatic apparatus necessary to complete DNA fragmentation [14]. However, subtle differences in intranuclear pH, or activating divalent cation concentration, affect the extent of DNA fragmentation [2]. DU145 and LNCaP cells do not completely fragment their DNA, or generate

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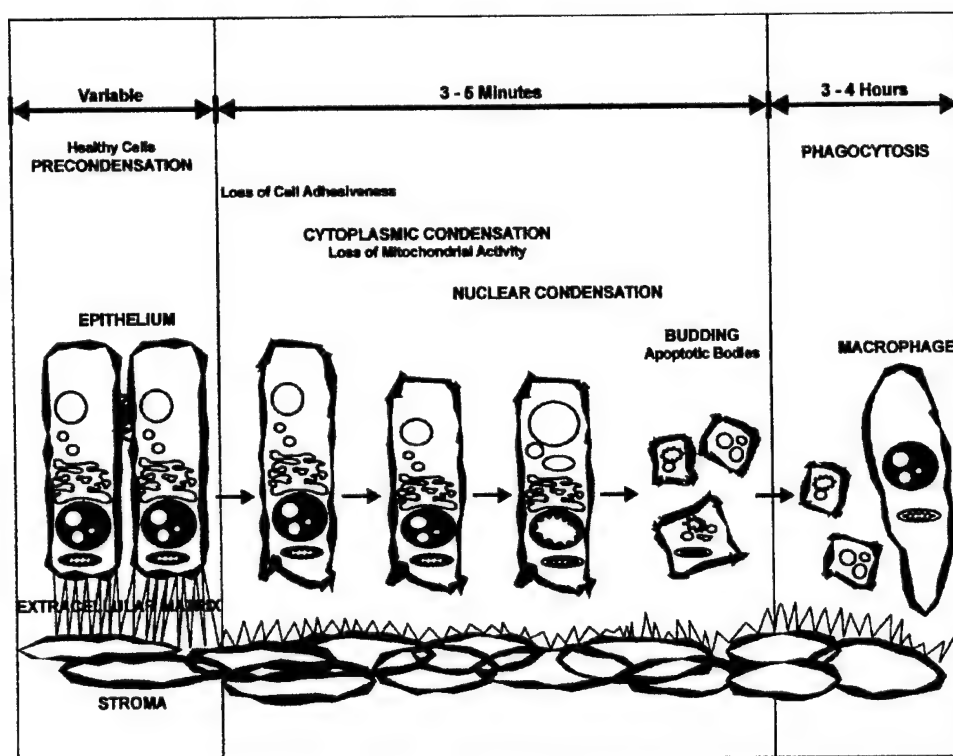


Figure 1. Schematic representation of stages of apoptosis in individual glandular epithelial cells. The process of apoptosis after hormone ablation in the prostate can be broken down into several stages. The length of the pre-condensation stage varies from cell to cell and probably reflects the micro-heterogeneity of hormone or growth factor in the environment. Cytoplasmic condensation involves the loss of the interactions between the dying cell and its epithelial neighbors, degradation of the extracellular matrix and the loss of mitochondrial membrane potential. During nuclear condensation, endonuclease activation results in the fragmentation of the DNA and its marginalization to the nuclear periphery. The fragmentation phase is characterized by the formation of apoptotic bodies, which are phagocytosed by the neighboring epithelial cells or resident macrophages.

oligonucleosome ladders after treatment with anti-androgens, even though the necessary enzymes are present in the nucleus [32].

2. *Annexin-V Staining:* In apoptotic cells the relocation of phosphatidylserine, from the inner to the outer leaflet of the cell membrane can be detected by Annexin-V. Programmed cell death in most prostate cancer cell lines can be assessed by measuring the number of cells stained positive for fluorescein-conjugated Annexin-V using fluorescence microscopy or flow cytometry [17, 25].
3. *Caspase Activation:* Activation of caspase-3, 6, 7, 8 and 9 plays a central role in apoptosis and can be used as a surrogate marker for apoptosis (Fig. 2). The activation of individual caspases is cell-type specific and also dependent on the type of apoptosis-inducing agent (11, 30).

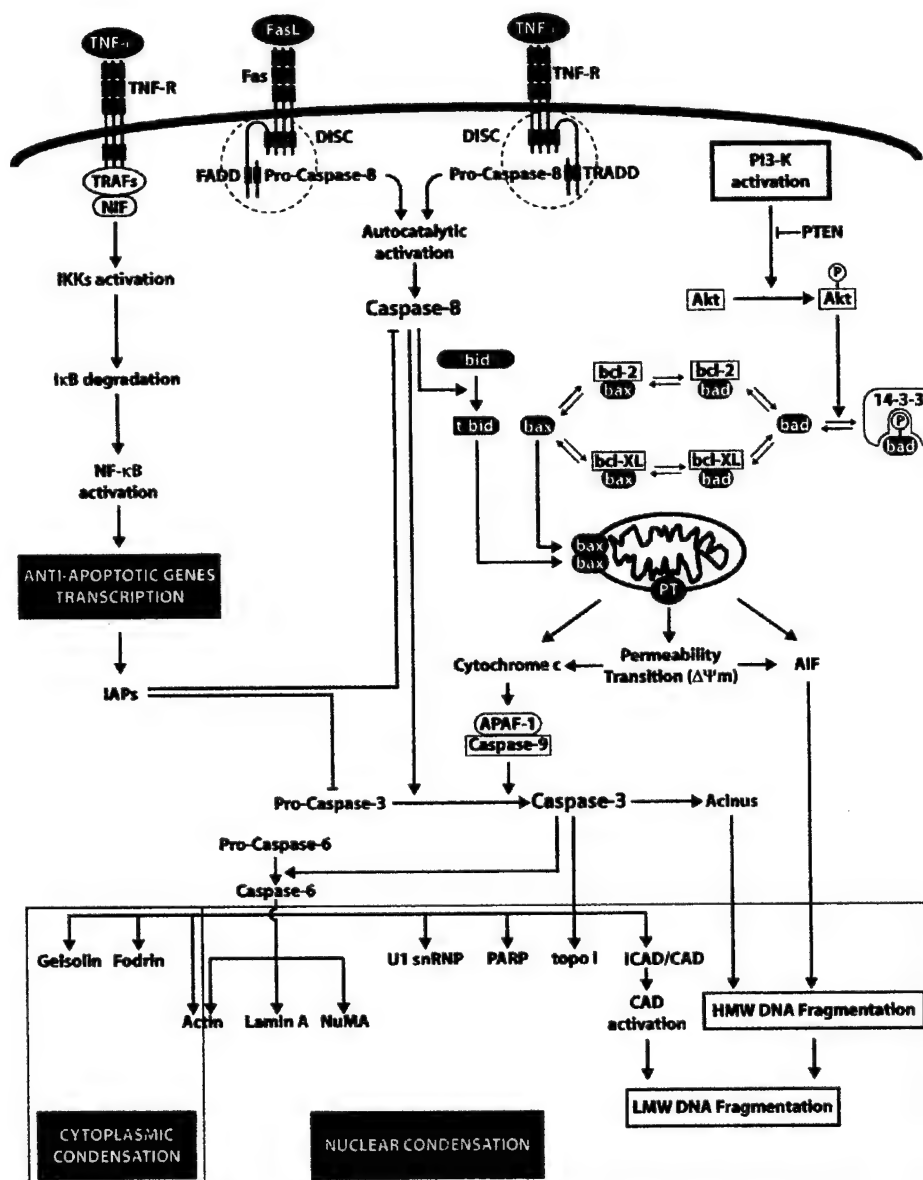


Figure 2. Extrinsic and intrinsic pathways of cell death and survival. The DISC (death-inducing signaling complex) is assembled after ligand binding to the death receptor. Autocatalytic activation of caspase-8 leads to activation of the executioner caspases and proteolysis of many substrate proteins. Activated caspase-8 also triggers the mitochondria-mediated pathway through the facilitated assembly of the mitochondrial permeability transition (PT) pore complex. This results in the dissipation of mitochondrial membrane potential ($\Delta\psi_m$), and release of cytochrome *c* and AIF from the mitochondria. The mitochondrial pathways leading to permeability transition are negatively regulated by Bcl-2 and Bcl-xL. Cytochrome *c* forms the apoptosome with Apaf-1 (apoptotic protease-activating factor 1) and pro-caspase 9, activating caspases 3 and 6, triggering high molecular weight (HMW), DNA fragmentation (through AIF and acinus) and oligonucleosome formation through CAD (caspase activated DNase).

Survival Pathways in Prostate Cancer Cells

Prostate cancer cell lines block apoptosis using a variety of survival mechanisms including PTEN, NF κ B, IAPs and Bcl-2 family proteins, which makes it difficult to generalize the effects of cytotoxic drugs based on a single cell line. For example the ALVA-31, PC3 and DU145 cell lines are highly sensitive to apoptosis induced by TRAIL (TNF-related apoptosis-inducing ligand) through the death receptor-mediated pathways, while LNCaP cells are resistant to TRAIL (19, 28). 45

1. PTEN

PTEN (phosphatase and tensin homologue deleted on chromosome 10, MMAC1/TEP1) is a tumor suppressor gene that functions as a dual-specificity phosphatase *in vitro* (12). Although PTEN gene is transcribed in both DU145 and LNCaP cells, the protein is only active in DU145 cells due to a frame-shift mutation in the PTEN gene in LNCaP cells that is also common to malignant prostate cancer (3, 28). PTEN dephosphorylates the lipid signal transduction molecule phosphoinositide 3,4,5-trisphosphate [PI(3,4,5)P₃] and serve as a negative regulator of the signaling events mediated by phosphatidylinositol 3-kinase (PI3-K) (13). Cell lines, such as the LNCaP, lacking PTEN phosphatase activity display constitutively elevated steady-state levels of PI(3,4,5)P₃ which activates PDK1 and PDK2. This results in constitutive activation of Akt (kinase B), a survival promoting serine/threonine kinase. Intracellular signaling through this pathway suppresses apoptosis by phosphorylating and inactivating caspase-9 and pro-apoptotic Bcl-2 family member Bad (6) (Fig. 2). The loss of PTEN expression in LNCaP cells elevates Akt activity and protection from TRAIL-induced cell death by sequestering Bad from the mitochondrial membrane and blocking the activation of the executioner caspases (21). However, this regulation of the PI3-K/Akt pathway is both death receptor and cell-type specific, as the lack of PTEN expression does not render LNCaP cells insensitive to TNF or PC3 cells insensitive to TRAIL (9). 50 55 60 65

2. NF- κ B and IAPs

Execution of the apoptotic pathways is also regulated by the transcription factor, NF- κ B, which is normally found as an inactive cytoplasmic heterodimer complexed with its cognate inhibitor, I κ B (1). Multimerization and recruitment of TRAFs (TNF receptor-associated factors) to the cell surface receptor along with NF- κ B-inducing kinase (NIK) activates the I κ B kinases (IKK α and IKK β). This leads to the phosphorylation, polyubiquitination and degradation of I κ B (16, 31), and the release of NF- κ B which translocates to the nucleus to activate transcription of the genes responsible for the anti-apoptotic response (4). The IAP proteins suppress apoptosis by binding to and inhibiting the proteolytic activity of caspase-3, -7, and -9. The IAPs also bind to TRAF hetero-complexes, interfering with the upstream activation of pro-caspase-8 (22, 29). The activation of NF- κ B and the induction of IAPs is an integral part of the negative-feedback-control of the apoptotic machinery that protects cells from undergoing apoptosis. NF- κ B is constitutively active in several androgen-insensitive prostate adenocarcinoma cell lines including DU145, PC-3, Du-Pro and TSU-Pr1, but is not in androgen-responsive cell lines such as LNCaP and CWR22RV1 (26). In cells that display constitutive activation 70 75 80

of the IKK complex and enhanced expression of IAPs (DU145 and PC-3 cells), lowered sensitivity towards etoposide is displayed, compared to LNCaP cells, which are very sensitive to the drug and activate NF- κ B normally (15, 26). This suggests that the differences in NF- κ B and IAP activation pathways may be responsible for the differential sensitivity of prostate cell lines to chemotherapeutic agents. 85

3. *Bcl-2* Family

Anti-apoptotic members of the Bcl-2, such as Bcl-2 and Bcl-xL, block the cell death process through their protective role in mitochondrial integrity (Fig. 2) (10). These proteins heterodimerize and neutralize the pro-apoptotic activity of other Bcl-2 family proteins such as Bax and Bad. They also protect the mitochondria against the loss of permeability transition and block the release of cytochrome *c* and AIF (apoptosis inducing factor) from the intermembrane space to the cytosol. Bcl-2 overexpression is correlated with the emergence of hormone refractory disease and the progression of metastatic disease in prostate cancer (5). However, there is no correlation between endogenous Bcl-2 levels and androgen-responsiveness in different prostate cancer cell lines (8, 18, 27), nor is there a correlation between endogenous Bax expression levels and the status of androgen-sensitivity since only DU145 has been shown to be Bax-deficient (7). Thus, abrogation of the Bcl-2-regulated mitochondrial-mediated pathway is not entirely responsible for the emergency of androgen-resistance phenotype after hormone therapy. 90 95 100

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APPENDIX FOUR

Emergence of Metastatic Hormone-Refractory Disease in Prostate Cancer after Anti-Androgen Therapy

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Abstract The anti-androgens used in prostate cancer therapy have been designed to interfere with the normal androgen receptor (AR)-mediated processes that ensure prostate cell survival, triggering tumor cells to undergo programmed cell death. While anti-androgens were originally designed to treat advanced disease, they have recently been used to debulk organ-confined prostate tumors, to improve positive margins prior to surgery, and for chemoprevention in patients at high risk for prostate cancer. However, tumors treated with anti-androgens frequently become hormone refractory and acquire a more aggressive phenotype. Progression toward metastatic hormone-refractory disease has often been regarded as the outgrowth of a small number of hormone-independent cells that emerge from a hormone-dependent tumor during anti-androgen treatment by natural selection. While a number of selective advantages have recently been identified, there is also considerable evidence suggesting that the progression toward metastatic hormone-refractory disease is a dynamic process which involves abrogation of programmed cell death as a result of the attenuation of DNA fragmentation and maintenance of mitochondrial membrane potential in tumor cells; the upregulation of stromal-mediated growth factor signaling pathways; and the upregulation of extracellular matrix (ECM) protease expression. *J. Cell. Biochem.* 9999: 1–9, 2004. © 2004 Wiley-Liss, Inc.

Key words: XXX^{Q3}; XXXX

ANTI-ANDROGENS IN PROSTATE CANCER THERAPY

Anti-androgens such as Casodex have been designed to trigger androgen-dependent tumor cells to undergo programmed cell death [Furr, 1996; Furr and Tucker, 1996]. Casodex is now being used as a monotherapy due to its preservation on testosterone levels and sexual potency and has provided an attractive alternative therapeutic approach to surgical intervention [Kolvenbag and Nash, 1999]. However, tumor treated with anti-androgens frequently become hormone refractory and have an increase propensity for metastasis [Grossmann et al., 2001; Kish et al., 2001; Knox and Moore,

2001; Rubben et al., 2001]. Resistant to anti-androgen therapy has been attributed to the intrinsic ability of tumor cells to abrogate the cell death process induced by the anti-androgens. In particular, the progression toward hormone-refractory disease has been related to androgen receptor (AR) status as evidenced by the strong correlation between AR expression and metastatic progression of prostate cancer both in vitro and in vivo [Tilley et al., 1990]. It has also been suggested that failure of conventional androgen deprivation therapy in prostate cancer is caused by the clonal expansion of tumor cells that continue androgen-dependent growth despite of low concentrations of serum androgens through the amplification or mutation of the AR gene [Koivisto et al., 1997]. However, both normal and mutated AR gene expression and amplification have been shown in androgen-insensitive tumors [Wallen et al., 1999; Haapala et al., 2001]. In addition, while Casodex down-regulates nuclear AR level in prostate cancer cells through translocation to the cytoplasm and proteosomal-mediated degradation, transcription of the AR gene does not appear to be altered significantly [Waller et al., 2000; Lee et al., 2003]. Thus, neither mutation

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nor changes in expression of AR can fully account for the loss of androgen-responsiveness and the increased malignancy of prostate cancer cells. Overexpression of *Bcl-2* gene has also been related to the metastatic progression of various cancers, including hormone refractory prostate tumors [Colombel et al., 1993]. However, overexpression of *Bcl-2* fails to attenuate Casodex-induced programmed cell death in prostate cancer cells [Lee et al., 2003], suggesting that the increased *Bcl-2* expression seen in advance stage prostate tumors is not necessarily the cause for the survival of hormone-refractory cells after anti-androgen therapy. Thus, it is likely that alterations in AR and *Bcl-2* expression result from mutations in cancer cells after the induction of hormone-independent tumor and do not initiate the progression to androgen independent disease.

MORPHOLOGICAL CONSIDERATIONS: THE RODENT PROSTATE

The rodent prostate gland is a complex arborized network of ducts, with a number of cell types which display anatomical and biochemical heterogeneity, and substantially different sensitivities to androgen ablation [Cunha et al., 1987]. The secretory epithelial cells are localized in the distal and intermediate regions of the ducts [Sugimura et al., 1986; Rouleau et al., 1990; Sensibar et al., 1991] and are critically dependent on androgens for survival [English et al., 1987]. In the proximal region of the ducts, the luminal epithelial cells display little if any secretory activity [Lee et al., 1990; Rouleau et al., 1990]. Neither these cells, nor the basal cells that are also localized to the proximal region, appear to require androgens for survival. The homeostasis of the prostatic gland is governed by the close reciprocal interaction between the stromal and epithelial tissue compartment [Chung and Cunha, 1983; Cunha et al., 1983; Chung et al., 1984; Miller et al., 1985]. Regression of the rodent prostate is initiated approximately 12 h after castration, when the level of 5 α -dihydrotestosterone falls below that needed to sustain survival [Isaacs, 1984]. The reduction in prostate size that occurs over the next 3–6 days is primarily due to the selective loss of the secretory luminal epithelial cells in the distal and intermediate regions, resulting in the complete obliteration of many of the ducts while maintaining the proximal segments of the ducts following castration

[English et al., 1987]. The loss of the secretory epithelium without the concomitant loss of stroma results in an increase in stromal-epithelial ratio [DeKlerk and Coffey, 1978]. The reduction of prostatic tumor size after treatment with anti-androgen is primarily due to the selective loss of androgen-dependent tumor cells, resulting in the substantial increase in the stroma-epithelium ratio [Hellstrom et al., 1997]. Since the stromal compartment of the tumor does not undergo significant apoptosis after hormone ablation, this results in a substantial increase in the stromal-epithelial ratio (Fig. 1). Thus, as the tumor cells die, the relative levels of the growth and survival factors secreted by the stroma increases substantially within the tumor.

MORPHOLOGICAL CONSIDERATIONS: HETEROTYPIC INTERACTIONS IN PROSTATE TUMORS

The survival of tumor cells is also dependent on the interaction with the prostate stroma and ECM through integrin and growth factor-receptor-mediated systems [Cunha et al., 2002; Sung and Chung, 2002]. The interaction between these growth factors including, but not limited to, insulin-like growth factor (IGF), epidermal growth factor (EGF), and members of the fibroblast growth factor families (FGF) such as FGF2, FGF7 and FGF10 and their cognate receptors [Mori et al., 1990; Cohen et al., 1991; Chung et al., 1992; Comoglio and Trusolino, 2002] requires both the expression of the receptor in the tumor cells and the expression and secretion of the ligands from the stroma as well as defined components of the ECM, including chondroitin sulfate and heparan sulfate [Kan et al., 1993]. While their cognate receptors are expressed in the luminal or basal epithelial cells, growth factors such as IGF [Cohen et al., 1991], EGF [Cohen et al., 1994; Freeman et al., 1998], and FGF [Story, 1995; Story et al., 1989; Sherwood et al., 1992; Alarid et al., 1994] are expressed and secreted primarily by the stromal compartment of the prostate. An increase in stroma-epithelium ratio in the prostate tissue thus leads to an increase in local concentration of these growth factors in the tumor cells, activating signaling pathways essential for both proliferation and survival of tumor cells. Increased localization of growth factors such as IGF [Chan et al., 1998a,b; Mantzoros et al.,

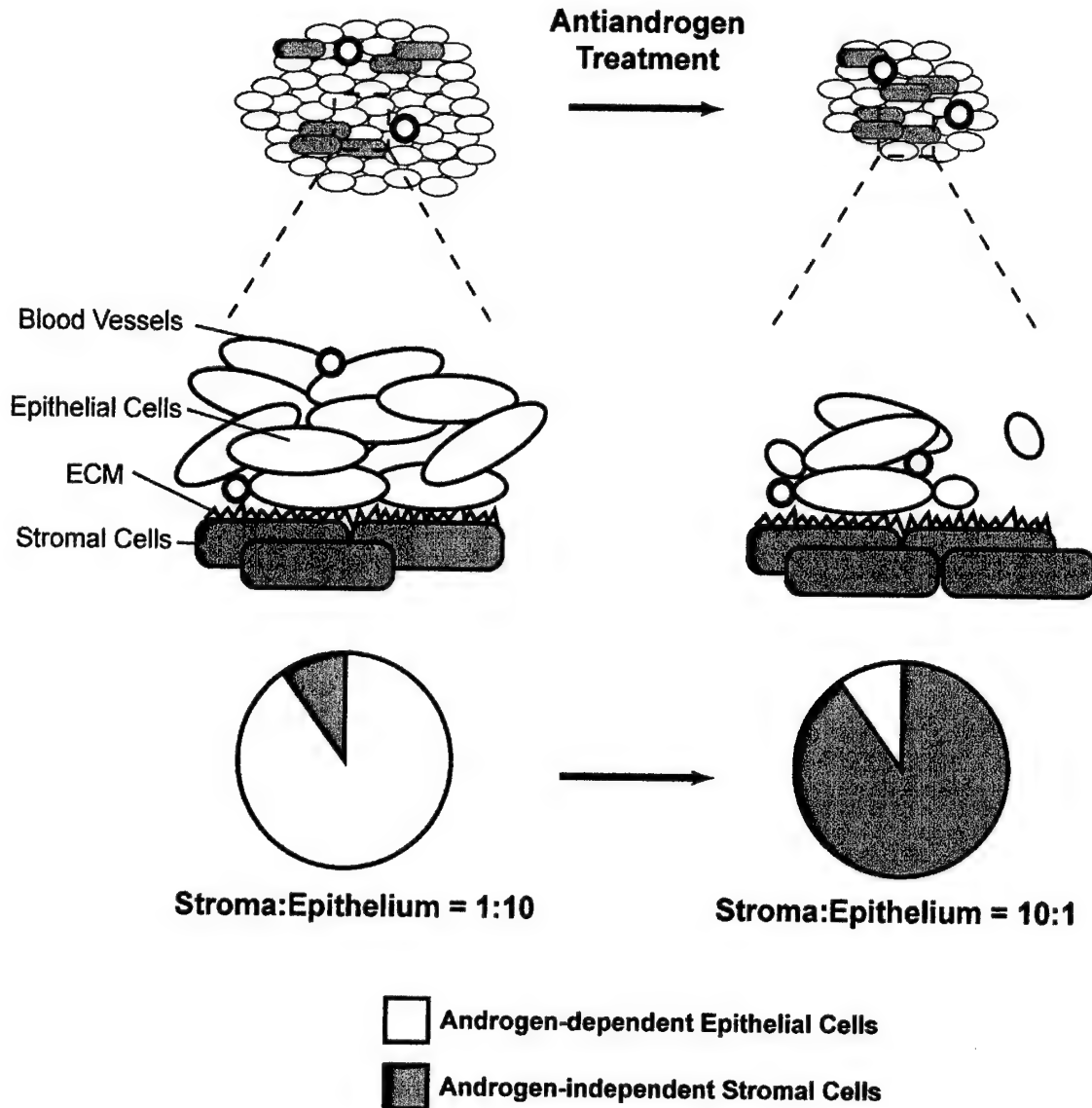


Fig. 1. Changes in stromal-epithelial ratio of prostate tumor after anti-androgen therapy. The reduction of prostate tumor size after anti-androgen therapy is primarily due to the selective loss of androgen-dependent tumor cells, which undergo apoptosis. Since the stromal compartment of the prostate tumor does not require androgens for survival, this results in a substantial increase in the stromal-epithelial ratio after treatment with anti-androgens.

1997; Wolk et al., 1998], EGF [Connolly and Rose, 1990; Hofer et al., 1991; Carruba et al., 1994; McEleny et al., 2002], and FGF [Nakamoto et al., 1992; Cronauer et al., 1997; Dorkin et al., 1999] in the prostate tumor epithelium has been correlated with the metastatic progression of prostate cancer [Sciavolino and Abate-Shen, 1998]. Changes in the relative levels of these growth factors or glycosaminoglycans can influence the induction of apoptosis in glandular epithelial cells and contribute to

the cellular micro-heterogeneity during tumor progression [Sugimura et al., 1986; Cunha et al., 1987]. This dynamic interaction between the tumor cells, stroma and ECM is required and responsible for the activation of ras- and PI3-K-dependent signal transduction pathways that appear to be essential for tumor cell survival [McKeehan, 1991; Yan et al., 1992].

Hence, the activation of proliferation and survival pathway by stromal-derived growth factors may override apoptotic-signaling pathways

and render the prostate tumor cells resistant to apoptosis. Moreover, activation of some of these pathways may also promote tumor cell motility, angiogenesis and metastases, suggesting that while anti-androgens may selectively induce cell death of the tumor cells, the resultant increase in the stromal-epithelial ratio may ultimately lead to tumor cell survival and metastatic progression.

FEATURES OF CASODEX-INDUCED CELL DEATH

Mitochondrial Disruption

In contrast to the normal sequence of apoptosis of prostatic epithelial cells after androgen ablation, Casodex induces the loss of cell adhesion prior to the loss of mitochondrial activity [Lee et al., 2003]. Casodex also induces the release of cytochrome *c* without disrupting mitochondria membrane potential ($\Delta\Psi_m$), agreeing with the recent suggestions that the release of cytochrome *c* and disruption of $\Delta\Psi_m$ are separate events in the processes leading to mitochondrial disruption [Achenbach et al., 2000; Loeffler and Kroemer, 2000; Grubb et al., 2001]. Since the release of cytochrome *c* alone does not necessarily lead to cell death in prostate cancer cells [Carson et al., 2002], this suggests that a small but significant portion of Casodex-treated prostate cancer cells can abrogate the death process and remain viable after the loss of cell adhesion.

DNA Fragmentation

Another important feature of Casodex-induced programmed cell death in prostate cancer cells is the lack of low molecular weight (LMW) DNA fragmentation or DNA laddering [Zhan et al., 2002]. Although often regarded as one of the major hallmarks in programmed cell death that occurs during nuclear condensation, DNA fragmentation (especially DNA laddering) is not seen in all cells types undergoing apoptosis, and is clearly not necessary for apoptotic cell death. While most prostate cancer cells appear to have the enzymatic apparatus necessary to complete DNA fragmentation [Marcelli et al., 2000], subtle differences in intranuclear pH, activating divalent cation or inhibiting monovalent cation concentrations may attenuate LMW DNA fragmentation in programmed cell death [Barry and Eastman, 1992]. Regardless of the mechanism of the abro-

gation of LMW DNA fragmentation, Casodex appears to induce delayed DNA fragmentation which may provide a selection advantage for the emergence of hormone-refractory disease.

Expression of Extracellular Matrix Proteases

During programmed cell death, expression of extracellular matrix (ECM) proteases is induced. The degradation of the ECM is required for the loss of cell-substratum interaction and the apoptotic elimination of superfluous or damaged cells. Casodex also induces a dose-dependent increase in several ECM proteases, including MMP-2 and Cathepsin B during the induction of programmed cell death in prostate cancer cells [Zhan et al., 2002]. Many, if not all, of the ECM proteases that are induced in dying cells are also expressed by metastatic tumor cells and have been associated with the invasive phenotype of these cells [Liotta and Stetler-Stevenson, 1990; Sloane, 1990]. Thus, Casodex-induced up-regulation of pro-invasive ECM proteases during cell death may render the surviving tumor cells that fail to fragment their DNA more invasive and more metastatic.

Stromal-Induced TGF β Pathway and Metastatic Progression

In normal prostate, the isoforms of transforming growth factor beta (TGF β I-III) are expressed in both stromal and epithelial cells and functions as growth inhibitors [Story et al., 1993, 1996]. Both TGF β receptors (TGF β -RI and TGF β -RII) are abundantly expressed in normal prostate epithelial cells and appeared to be downregulated and exhibit progressive reduction of expression in primary cancer, and lymph node metastases [Guo and Kyprianou, 1999]. In malignant prostate, TGF β appears to inhibit the immune response and promote angiogenesis, ECM deposition, and metastases [Wilding, 1991; Steiner, 1995]. We have recently shown that increases in stroma volume within prostate tumors lead to a decrease in localization of TGF β -RI in the tumor cells and an increase in TGF β -III in the tumor epithelium (unpublished data), suggesting that the increase in stromal-epithelium ratio after treatment with anti-androgens induces a metastatic phenotype by increasing the level of TGF β , abrogating its inhibitory affect on cell growth by decreasing the level of TGR β receptors in the prostate tumor epithelium.

Emergence of Hormone-Refractory Metastatic Diseases

Other than mitochondrial disruption and DNA fragmentation, which complete the death process, dying and metastatic cells share an

astounding number of similarities. The delayed and incomplete fragmentation induced by Casodex in prostate cancer cells may provide an opportunity for extensive, but inappropriate DNA repair, leading to genomic instability. Because a small portion of non-adherent

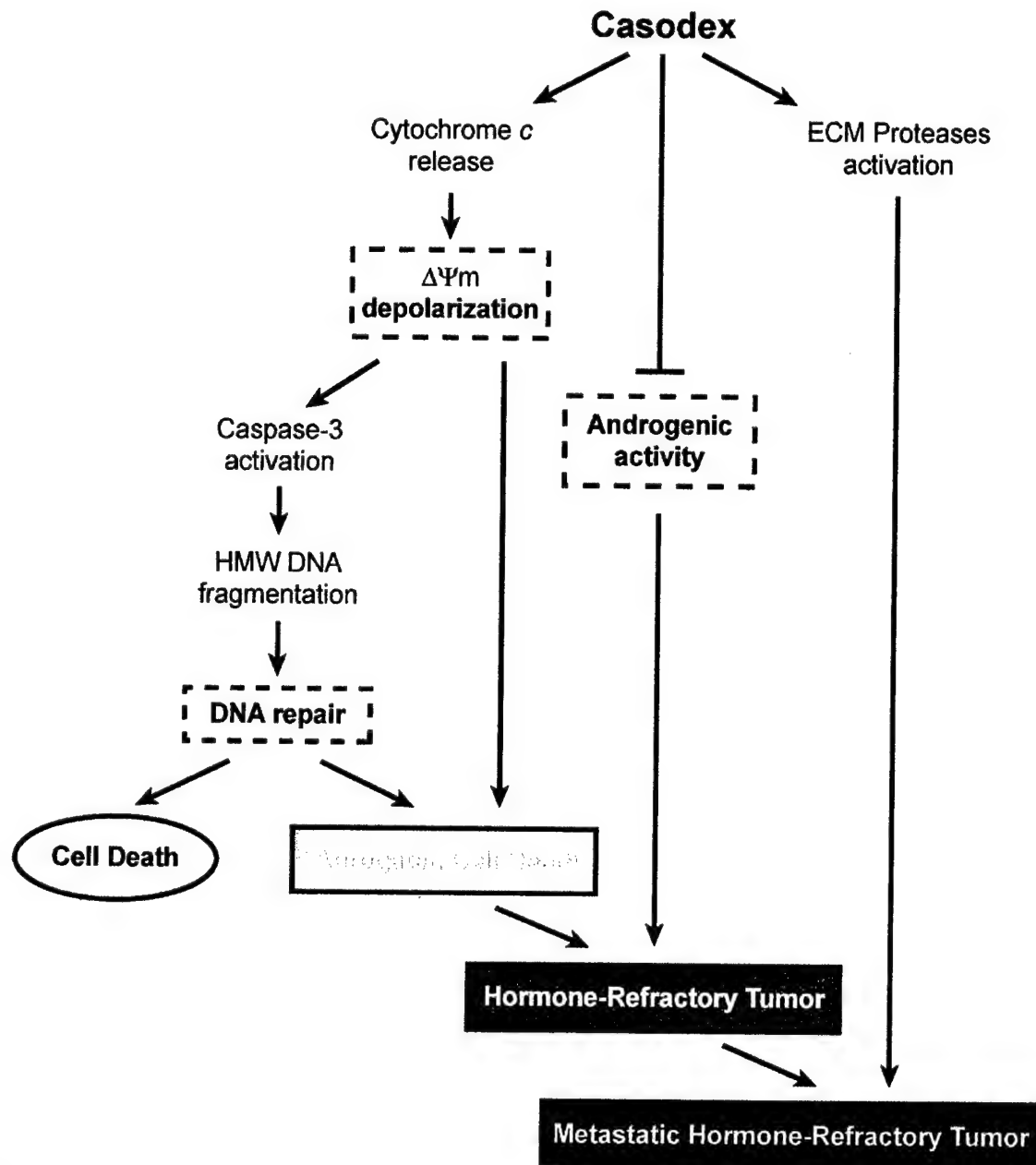


Fig. 2. Intrinsic factors for the emergence of metastatic hormone-refractory disease in prostate cancer after treatment with Casodex. Casodex triggers the programmed cell death processes in prostate tumor cells by interfering with normal androgen receptor (AR)-mediated cell survival process. However, Casodex induces cell death without disrupting $\Delta\Psi_m$ and results in an extended lag phase of cell survival between the

initiation of cell death and the fragmentation of DNA. During this time, extensive but inappropriate DNA repair process may occur, producing genomic instability. While only a small portion of these hormone-refractory, genomic unstable tumor cells remains viable, Casodex-induced dose-dependent up-regulation of pro-invasive genes such as ECM proteases also renders them metastatically competent.

prostate cancer cells after Casodex treatment remains viable, Casodex-induced dose-dependent up-regulation of pro-invasive genes such as ECM proteases may also render them also capable of becoming metastatic (Fig. 2).

The microenvironment surrounding the prostate adenocarcinoma cells after treatment with anti-androgens may also play an important role in inducing an invasive phenotype in these cells that abrogate the apoptotic process. As discussed above, the increase of stromal-epithelium ratio in the prostate tumor after anti-androgen therapy increases the local concentration of a number of growth factors and activates proliferation and survival signaling pathways that override the apoptotic pathways induced by the anti-androgen. Upregulation

of stroma-mediated growth factor signaling pathways such as TGF β may promote cell motility and angiogenesis and contribute to the induction in metastatic potential. Thus the combination of the intrinsic factors from anti-androgen-induced cell death and extrinsic factors from the microenvironment surrounding these prostate adenocarcinoma cells contributes to the emergence of hormone-refractory metastatic diseases.

This is in contrast to the conventional view that resistance to anti-androgens is simply an outgrowth of a small number of hormone-independent cells that emerge from a hormone-dependent tumor during anti-androgen treatment through natural selection. Rather, this hypothesis states that the progression toward

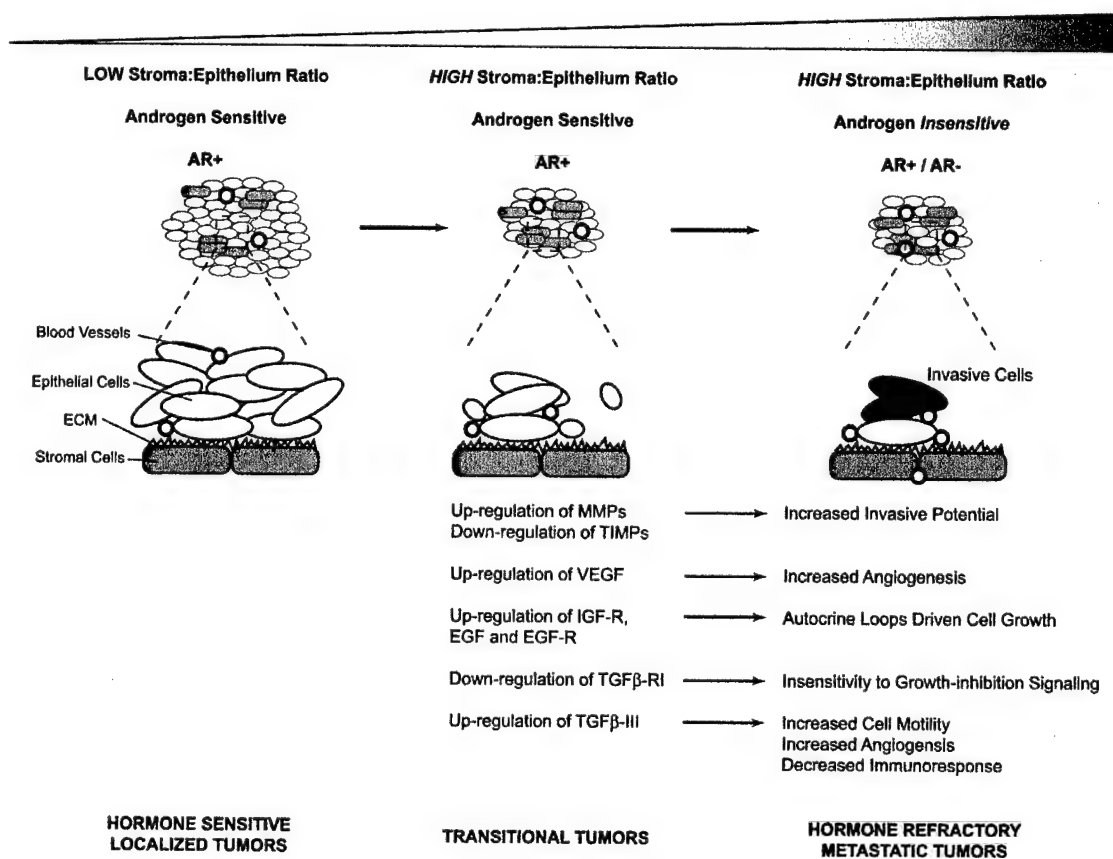


Fig. 3. Schematic diagram of the proposed molecular basis for the emergence of metastatic hormone-refractory disease in prostate cancer after anti-androgen therapy. Anti-androgen reduces prostate tumor size by selectively inducing programmed cell death of AR-positive androgen-sensitive tumor cells and increases the stromal-epithelium ratio within the tumor. The increase in stromal-epithelium ratio leads to the up-regulation of VEGF and TGF β -III, which enhances the growth of prostate

tumors by inducing cell motility and angiogenesis and down-regulation of TGF β -RI in the tumor epithelium that renders the cells insensitive to growth-inhibition signals. The increase in stromal-epithelium ratio also leads to an upregulation of stromal-mediated growth factor signaling pathways such as the IGF and EGF axis, which further promotes the cell proliferation and survival process and renders the tumor insensitive to androgens.

metastatic hormone-refractory disease is a dynamic process which involves abrogation of programmed cell death as a result of the attenuation of DNA fragmentation and maintenance of mitochondrial membrane potential in tumor cells; the upregulation of stromal-mediated growth factor signaling pathways; and the upregulation of ECM protease expression. This also suggests that the use of anti-androgen in prostate cancer therapy is bound to fail so long as it only targets adenocarcinoma cells in the tumor while ignoring the influence of the microenvironment of the prostate tumor (Fig. 3). This highlights the need to understand the role of the reactive stroma in the maintenance of cell survival after anti-androgen therapy and the need to identify new drug targets to block the dynamic effects of the stroma during anti-androgen therapy.

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APPENDIX FIVE

Uncorrected Draft

Comparative Functional Genomics: Analysis of changes in mRNA profiles in multiple model systems for understanding basic biological phenomenon.

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Abstract

We are interested in the molecular conditions within cells that sensitize them to active cell death (apoptosis). This is important in the study of cancer and other biological conditions because by understanding what makes cells die normally, we will better understand how to deal with cells where these processes are disrupted. We have taken a gene macroarray approach to analyzing changes in gene expression in breast cancer cells in culture when they are treated with Tamoxifen. However, rather than using the pairwise, n-fold comparison of mRNA abundances that has become standard with array analyses, we are using what we term a comparative functional genomics approach. With this approach, we are looking for consistent changes in gene expression that are conserved in multiple models of inducible apoptosis. In the current study, we have examined breast cancer cells treated with TAM, ICI and TNF α and prostate cancer cells treated with Casodex. Rather than arbitrarily defining a n-fold value as being significant, we are relying on concordant changes in the gene expression profiles in the different models to define which changes have a potential biological significance. In our pilot study, we designed a small array experiment and have identified four genes that may be relevant for active cell death sensitivity associated with Tamoxifen blockage of estrogen receptors.

Introduction

The current approach to the analysis of genomics and proteomics data is complex. Gene array analyses and 2D protein gels experiments produce massive amounts of data that must be interpreted to identify those pieces of data that are significant. One ends up with a very large picture of what is happening, but it is often confusing as to which part of the picture is the most important. When zoologists were attempting to unravel vertebrate anatomy, an innovative comparative vertebrate anatomy approach was developed to study isolated structural systems. We believe that a similar comparative approach can be applied to array analysis to reveal biological significance intrinsic to gene expression level profiles with respect to isolated biological events.

We are interested in a single biological phenomenon, the process of apoptosis. We have been using a differential screening approach to identify genes that are differentially expressed in an apoptotic-induced state as compared to a normal state. This approach is useful because the profile of gene expression within a cell determines the behavior of that cell and the differences in gene expression defines differences in cell behavior. With respect to traditional differential screening of a cDNA library or array, it has become standard to set an arbitrary n-fold differential expression value as the criteria to decide what might be biologically significant between a pair of conditions (for example cells treated with a specific drug versus untreated cells). This approach does not consider that there may be changes in gene expression that are not involved in the process being studied. In addition, setting a high arbitrary criteria for the amount of change that is significant for gene expression does not allow one to consider that, in some cases, small changes in the expression of specific genes may be sufficient to have a biological effect.

We have developed a comparative functional genomics approach to identify changes in gene expression that are conserved between different model systems undergoing a common biological process, in our case apoptosis. By doing this, we are attempting to tease out the common events that define the apoptotic process by comparing consistent changes (sometimes subtle ones) that occur in these model systems. This is a different approach than traditional methods of analyzing functional genomic data. The comparative functional approach recognizes that if a biological process is used by all cells, then we can identify those genes that are important for that process by identifying which genes are regulated in a similar fashion by induction of the process in different systems. In the current manuscript we describe our pilot screen of a small array of genes for estrogen receptor mediated gene changes that are conserved in four cell culture model systems of apoptosis sensitivity.

Defining Apoptosis:

Apoptosis is an active cell death process that cells can activate as a response to certain endogenous or environmental stimuli (Lawen, 2003; Tenniswood *et al.*, 1992). This is a naturally occurring process that has been characterized in the removal of unwanted tissue during development and the removal of damaged cells (as reviewed in Metzstein *et al.*, 1998; Vaux, 2002). The apoptotic processes within a cell are controlled by biomolecular balances and switches. Because different cell types respond uniquely to environmental

stimuli and growing conditions, the cues to activate apoptosis may vary from cell type to cell type. However, because apoptosis is essentially ubiquitous process, the overall repertoire of receptors, signal transducers, activators and executors within all cells must be similar. The differences in the expression of these molecules are indicative of the tailor made balance for each cell type. Thus, apoptosis is an interesting biological process to study from a basic biological perspective as a system that can be controlled through a complex series of signaling events and a variety of inputs. It is also medically important from a number of perspectives including understanding and treatment of diseases where apoptosis is misregulated such as cancer.

Cancer and the apoptotic process:

Cancer cells arise from normal tissue through a series of modifications to biological cellular processes. These events include induction of cell cycle and down regulation of apoptosis as early events through to enhancement of interactions with and modifications to the extracellular environment in metastatic disease (Jechlinger *et al.*, 2002). Normally, when a cell becomes dysfunctional, apoptosis is induced and the cell dies for the overall benefit of the organism (Vaux, 2002). Pre-cancerous cells are dysfunctional in nature, and are able to survive because of modifications that make them more resistant to apoptosis induction.

The goal of cancer biology research is to find a way to kill cancer cells. Some chemotherapeutic drugs that have been designed to treat cancer do so by sensitizing these cells to apoptosis inducing signals. Of particular interest here are the drugs Tamoxifen (TAM) and ICI 182,780 (ICI), estrogen analogues that block estrogen receptors and the testosterone-analogue Casodex (CAS) that blocks testosterone receptors (Mandlekar and Kong, 2001; Wilson *et al.*, 1995; Veldsholte *et al.*, 1992).

Tumors and cell culture systems:

The majority of breast tumors are derived from mammary gland epithelial cells. These tumors start as ductal carcinoma *in situ* and progress through invasive carcinomas to metastatic disease (Russo *et al.*, 1998). Mammary epithelial cell survival is normally dependent upon the presence of estrogens. Many kinds of breast cancers derived from these cells are also dependent upon estrogen to enhance resistance to apoptosis. Estrogen analogues that bind intracellular estrogen receptors and prevent proper functioning of the receptor can either induce apoptosis or produce a cell that is more sensitive to apoptosis inducing signals (Mandlekar and Kong, 2001). In the current study, we recapitulate these effects in culture by treating MCF7 cells, an estrogen dependent human mammary epithelial cancer cell line with the estrogen analogues TAM and ICI.

Prostate tumors are generally derived from testosterone dependent prostate epithelial cells (de Marzo *et al.*, 2001; Morrissey *et al.*, 2002). Some prostate cancers and cancer derived cell lines are also dependent upon testosterone for normal function. Apoptosis sensitivity can be induced in these cells if the function of the testosterone receptor is blocked by a testosterone analogue that prevents proper functioning of the receptor. We mimic these effects in culture by treating PC346C cells, an androgen dependent human prostate tumor derived cell line, with CAS.

Cell culture systems are used in our analysis because the response of single cell types is difficult to detect and analyze *in vivo*. The use of cell culture systems allows us to detect small fluctuations in the mRNA abundance for specific transcripts without the masking effects of other cell types. We are also able to tightly control the environmental conditions to which the cells are exposed and eliminate tissue interactions and other variables inherent to *in vivo* studies that may confound the experiment. For each single model system, we use a comparison between cells treated with hormone receptor blocking drug and those that have not been treated to study how the molecular environment changes in response to drug induced apoptosis sensitivity. In the current study, the selected drug treatments induce an early response of apoptosis sensitivity or resistance. By comparing how MCF7 cells respond to TAM and ICI treatment and comparing these to the PC346C response to CAS treatment, we hope to discover genes that are regulated in a concordant fashion between these conditions. We have also included here a comparison to MCF7 cells treated with the apoptosis-inducing factor TNF α . This treatment sensitizes the cells to apoptosis through a non-hormone receptor mediated pathway and serves as an outlier for comparison. Those genes that are concordantly regulated by TNF α and the hormone receptor blocking treatments are more likely to represent genes involved in apoptosis sensitivity rather than some other side effect of the hormone receptor blockage.

In this study we compare two approaches to identify genes involved in apoptosis. The first approach is a standard n-fold based comparison of MCF7 cells treated with TAM to control treatment. The purpose of this experiment is to demonstrate n-fold analysis. The second approach is our proposed comparative functional genomics approach. Here we look for concordant regulation of gene expression based on absolute differences in gene expression levels. Any detectable difference in mRNA abundance is considered to be potentially biologically significance. Here we present data comparing the molecular response of hormone receptor blockage in estrogen dependent breast cancer cells with androgen dependent prostate cancer cells and with TNF α treated breast cancer cells. All treatments induce apoptosis sensitivity. In our comparisons the central focus is always MCF7 cells treated with the estrogen analogue TAM. As diagrammed in Figure 1A, genes that are regulated by TAM, ICI, CAS and TNF α are of a number of distinct classes. We are interested in those genes represented by the black area in the diagram, those genes that have concordant regulation in all four model systems. By comparing all four kinds of treatments, we are able to identify those genes that are most important for the apoptosis sensitivity because they are regulated in the same fashion in all the model systems studied.

Materials and Methods

Cell culture and FACS analysis:

The cell lines used in these experiments were MCF7 human mammary cancer and PC346C human prostate cancer cells. Both cell lines were grown and maintained using standard cell culture techniques and treated as described in Zhan *et al.* (2003). The estrogen analogues TAM and ICI were used at concentration of 5 μ M and 10 μ M

respectively. The testosterone analogue Casodex (Bicalutamide) was used at a concentration of 50 μ M. TNF α was used at a concentration of 1 μ M. Cell densities were determined by a spectrophotometry at an absorbance of 590 nm.

Macroarray analysis:

Plasmid stocks of the 96 clones used were taken at random from an archive of clones previously purchased from Research Genetics (Invitrogen, Carlsbad, CA). The clones represent genes involved in general housekeeping, cell cycle, apoptosis, cell stress, signal transduction, DNA repair and some novel genes. Each stock was plated for single colony isolates and plasmid was prepared using a spin mini-prep plasmid kit (Qiagen, Mississauga, Canada). Identities of the clones were confirmed using sequence analysis (DYEnamic ET terminator kit, Amersham Biosciences, Little Chalfont, UK).

PCR products were generated from the plasmid stocks using standard T3, T7 and SP6 primers whose sequences flank the cloning site as appropriate. These PCR products were separated by gel electrophoresis and purified using the QIAquick gel extraction kit (Qiagen, Mississauga, Canada). The purified PCR products were then double spotted onto nylon membrane (BioDyne B, Pall Corp., Port Washington, NY) using a 96 pin arraying device (V&P scientific, San Diego, CA). Sufficient copies of the arrays for the experiment were made at the same time. The DNA on the nylon membrane was denatured in 0.4 M NaOH for 1 min, neutralized in 0.5 M Tris-HCl, pH 7.5 for 1 min. The membranes were allowed to air dry and then the DNA was UV crosslinked to the membrane using a Stratalinker 1800 (Stratagene, La Jolla, CA). Protocols used to perform the array analysis are summarized in Figure 1B.

RNA preparation:

Media was removed from the cell cultures and replaced with Trizol reagent (Invitrogen, Carlsbad, CA). The Trizol reagent efficiently removed the cells from the plate and was transferred to polypropylene tubes and frozen. Once all of the cultures had been suspended in Trizol reagent, RNA isolation from all samples was performed as per the Trizol protocol.

cDNA:

Double stranded cDNA was made from total RNA using PowerScript-RT (BD Biosciences, Palo Alto, CA) and polyT primer. RNA from samples that had been grown with or without treatment for 48 hours were selected for analysis based on the TAM treated cell concentration results, suggesting that at 48 hours significant apoptosis is not yet occurring. The strand switching property of this RT was used to incorporate a complementary to universal primer sequence at the 3' end of the first strand cDNA. This universal primer was then used to initiate synthesis of the second strand using Taq polymerase. Random prime labeling was used to generate α^{32} P-dCTP labeled cDNA to that was used to probe the arrays. Unamplified cDNA was used to maximize the precision of the experiment at the expense of sensitivity.

Hybridization:

Arrays were screened with cDNA probes (MCF7 48 h control, MCF7 48 h TAM treated, MCF7 48 h ICI treated, MCF7 48 h TNF α treated, PC346C 48 h control and PC346C 48 h CAS treated). Prehybridization was performed using 14 ml Hybrisol II (Serologicals Corp., Norcross, GA) at 65°C for 2 hours. Hybridizations were performed in 7 ml Hybrisol II at 65°C for 18 hours. The hybridization solution contained 1.2 million DPM of probe per 7 ml of hybridization solution. Washes were done with the highest stringency being two 15 min washes with 0.1X SSC, 0.1% SDS at 65°C. Blots were stabilized in 2X SSC and wrapped in cellophane and exposed to autoradiography film (Hyperfilm MP, Amersham Biosciences, Little Chalfont, UK) and developed after a suitable period of exposure.

Data analysis:

Autoradiographs were scanned using an Epson 1660 Photo Perfection scanner. Images were straightened and cropped using Adobe Photoshop v7 for Windows. Calculation of background intensity, net spot intensity and volume in pixels were done using the histogram function in Adobe Photoshop v7. Analysis of these densitometry results was performed using Microsoft Excel v8 for Windows. A global mean method was used to normalize spot intensities between arrays. In brief, this was accomplished by generating a normalization factor for each array by totaling the net intensity values of all spots within each array and dividing the average for all arrays by each array total. The net intensity values for each spot on the array are then multiplied by the normalization factor for the array to achieve a normalized net intensity.

Results

Treatment with hormone receptor blocking analogues cause apoptosis sensitivity:

MCF7 cells treated with 5 μ M TAM for 96 hours show a dramatic reduction in cell number as compared to the control (Figure 2A). The number of cells after TAM treatment for 96 hours is approximately 30% of the number seen in the untreated control. The number of cells that persist after a 48 hour treatment is 73.2% of the number seen in the untreated control. Treatment of MCF7 cells with TAM for 48 hours results in the appearance of some apoptotic cells as detected by tunnel labeling and flow cytometry (Figure 3).

MCF7 cells treated with ICI gave similar results as those seen after TAM treatment (Figure 2B). These observations are consistent with the fact that the MCF7 cells are dependent upon estrogen for normal function and that both TAM and ICI are estrogen analogues that block the estrogen receptor. TNF α treatment of MCF7 cells also results in a dramatic reduction in cell number compared to control at 96 hours. When treated with the androgen analogue CAS, PC346C cells show a decrease in cell number as compared to controls (Figure 2B). In addition, it has been shown that MCF7 cells treated with TAM, ICI or TNF α and prostate cancer cells treated with CAS become

apoptotic (Swiatecka *et al.*, 2000; Nickerson *et al.*, 1997; Vanhaesebroeck *et al.*, 1993; Zhan *et al.*, 2003).

n-fold analysis of gene expression profiles:

Comparisons of mRNA expression profiles of TAM treated and control treated MCF7 cells for 48 hours were performed indirectly using custom-made macroarrays. A 48 hour treatment was chosen because it represents an early response state prior to the induction of apoptosis in the majority of the cells. The screen with both probes used in the differential screen identified 15 clones showing quantifiable levels of signal (Table 1). The detection of 16% (15/96) of the clones on the macroarray suggests that all of the clones with detectable signal represent genes that are abundantly expressed under these conditions.

A n-fold cutoff point of 1.5 fold, a state in which the hybridization levels in the treated sample is at least 50% higher or lower than the levels observed for the untreated sample, was used as the criteria to select out putative differentially expressed genes (Table 1). When TAM treated MCF7 cells are compared to control, the genes identified as differentially expressed would be ARHA, CACNA1F and PCNA. If the focus of our study had been to use n-fold analysis to identify differentially expressed genes, these would then be further studied for an involvement in the apoptosis phenotype.

As part of our study, we also used copies of the same macroarrays to analyze expression changes with different treatments of the MCF7 cells and PC346C cells (Figure 4). Although it wasn't our intention, the data has been analyzed using n-fold analysis (data not shown). For these treatments, at a n-fold cut off of 1.5 fold, the differentially expressed genes were as follows: (1) ICI treated MCF7 differential genes (HSPD1, EIF3S6, PCNA). (2) CAS treated PC346C differential genes (Hs.28426, ARHA, HSPD1, EIF3S6, TRA1). (3) TNF α treated MCF7 differential genes (Hs.28426, HSPD1, EIF3S6, TRA1). The expression levels in control and treated cell samples are shown in Figure 4.

Functional genomics comparison of gene expression profiles:

In our comparative functional genomics analysis, we look for any change in the expression levels of the genes on the array. In the set of 15 genes that are relevant to our analysis, all show a high level of expression in untreated cells. Thus a small difference in the expression level of these genes after treatment may not register as a significant change based on fold differences but could very well represent a significant difference when considering the number of active transcripts that may be present in the cells or in particular cells. The mechanisms that activate and inhibit apoptosis are carefully balanced. Small perturbations in the concentrations of molecules affecting this balance can have a cascading effect. Therefore any change in a molecule involved in apoptosis sensitivity can be biologically significant.

In Figure 5, we have categorized the comparison of treated as compared to control expression as up-regulated (green), down-regulated (red) or no difference (yellow). In the analysis of expression trends we find that there is a consistent increase in HSPD1, EIF4S6, and PCNA expression levels after drug treatment in all cases. We also find a consistent decrease in the expression of DDX51. We therefore consider this group of

gene responses as potentially involved in the apoptosis sensitivity phenotype that is common between all the treatment groups. IKBKB, RDH5, XRCC1 and CACNA1F are consistently regulated after TAM and ICI treatment of MCF7 cells, as well as CAS treatment of PC346C cells. We thus consider induction of these genes as a common hormone receptor mediated response. Expression of genes such as Hs.271783, MSH6, Hs.28426 are induced after TAM and ICI treatment of MCF7 cells, we consider the induction of these genes as an estrogen receptor mediated response.

Discussion

In the first part of our analysis, we show by a n-fold analysis at a 1.5 fold differential abundance cut-off value that PCNA, CACNA1F, and ARHA are differentially expressed as defined by a differential screen with TAM treated and untreated MCF7 derived cDNA probes. This is a simple analysis of how TAM changes the expression profile of MCF7 cells in culture. Traditionally, these three genes would be further examined to identify what their function might be in an apoptosis susceptible phenotype. With the comparative functional genomics approach, we are assuming that any changes in gene expression at any level must have some biological significance if they are conserved in different model systems induced to undergo the same biological process. We are therefore looking for consistent up-regulation or consistent down-regulation of genes after exposure to different stimuli (different drug treatments) that produce similar phenotypes (cells with ACD sensitivity) in different inducible apoptotic models. By looking at a second estrogen analogue (ICI) that also blocks the function of the estrogen receptor, we develop the criteria to be able to define which genes might underlie an estrogen receptor mediated response including sensitivity to death signals. When we examine the response of PC346C cells to CAS to our list of model systems we expand our study to be able to address which genes are common in more generalized hormone receptor blockage responses. Finally when we examine the effects of a $\text{TNF}\alpha$, which induces apoptosis via cell surface receptors and a series of signal transduction cascades, we identify those genes that mediate common responses between these treatments and therefore more likely to be involved in the common apoptosis sensitivity phenotype. The multi-model comparative approach thus allows us to develop certain insights that a simple two-way differential analysis cannot. Given the diversity of the model systems we can begin to unravel those molecular events that underlie a receptor mediated apoptosis sensitivity phenotype.

Our focus for all the comparisons has been MCF7 cells treated with TAM. With the n-fold analysis PCNA, ARHA and CACNA1F were identified as differentially regulated by TAM treatment. The role of PCNA in apoptosis is discussed below. ARHA is primarily involved in actin cytoskeletal rearrangements and metastatic invasion in response to integrin activation (Cannizzaro *et al.*, 1990; Moreau *et al.*, 2003; Schoenwaelder *et al.*, 2002). ARHA activation and inactivation has also been shown to have a role in inducing apoptosis therefore it is questionable whether ARHA activation is directly related to apoptosis (Debreuil *et al.*, 2003; Moore *et al.*, 2003). The role of ARHA as an oncogene may be more related to invasion and metastasis rather than apoptosis regulation. CACNA1F is a type II voltage dependent calcium ion channel and

is expressed *in vivo* almost exclusively in eye tissues (Fisher *et al.*, 1997; Koschak *et al.*, 2003). It is therefore unlikely to be important in a ubiquitous process such as apoptosis.

If we compare the list of n-fold analysis of differentially expressed genes with TAM treatment with the results of our comparative functional genomics analysis, we find that only PCNA is on both lists. HSPD1, EIF3S6 and DDX51 show similar changes in expression patterns in all the model systems examined but were not identified in a differential screen for responses to TAM treatment.

The known functions of PCNA, EIF3S6, HSPD1 and DDX51 are consistent with a role in an ACD phenotype. PCNA is a protein that is regulated by cell cycle and is related to DNA repair (Bravo, 1986; Hoege *et al.*, 2002). It is involved in DNA replication during S-phase and is ubiquitinated during DNA repair. PCNA has also been associated with cancer phenotypes that has not been completely described, but likely relates to its role in DNA repair or regulation of the cell cycle. It has also been shown that cells that are actively proliferating can be induced to enter apoptosis by blockage of the cell cycle, therefore PCNA upregulation in response to hormone receptor blocking treatment may help to sensitize these cells to apoptosis (Wyllie, 1993). Eif3S6 is a protein involved in the initiation of translation and as such could be involved in modulating proteome profiles within the cell (Asano *et al.*, 1997). Changes in the levels of Eif3S6 could change which mRNA transcripts are selected for translation. EIF3S6 has been associated with breast cancer because EIF3S6 is a common site for the integration of the MMTV virus resulting in mammary tumors in mice (Asano *et al.*, 1997; Miyazaki *et al.*, 1997). HSPD1 is a heat shock protein that acts as a mitochondrial molecular chaperone and is the homologue of GroEL from *Escherichia coli* (Jindal *et al.*, 1989). The consistent upregulation of HSPD1 with the treatments as shown in Figure 5 suggests that these treatments may be inducing cellular stress within the mitochondria of these cells. Mitochondrial instability has been associated with TAM treatment (as reviewed in Mandlekar and Kong, 2001). DDX51 has only recently been described (Strausberg *et al.*, 2002). DDX51 contains a DEAD box (Asp-Glu-Ala-Asp) sequence motif that has been associated with genes involved in DNA binding, DNA replication and transcription initiation (as reviewed in Rogers *et al.*, 2002). If Ddx51 also has an effect on these cellular processes then changes in the level of its expression could mediate changes in the molecular environment.

Conclusions:

This study has described two key observations. The first is that of the genes that have been spotted on these arrays, PCNA, EIF3S6, HSPD1 and DDX51 could have important roles in the apoptosis sensitivity associated with receptor mediated responses in breast cancer cells and prostate cancer cells. This role is supported by what is already known about these genes and their roles in apoptotic cellular phenotypes. Second, and more importantly, we present a novel way to examine and dissect gene expression and provide a novel comparative functional genomics strategy to assay for genes that may be relevant to a specific biological process. The genes identified by our comparative approach may not necessarily be identified through more traditional differential approaches at gene selection. Although the role of the genes identified in our study need further functional

characterization, we feel that comparative functional genomics approach is potentially useful for identifying genes involved in apoptosis and has broader applicability to other biological processes.

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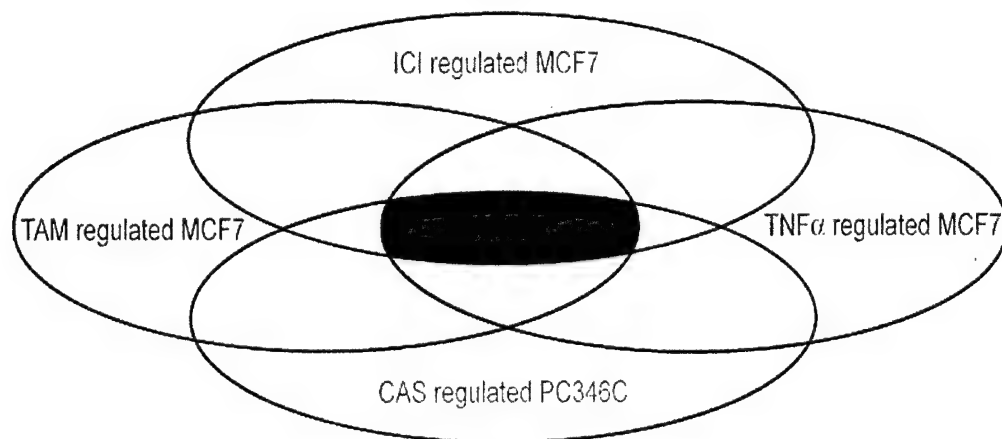
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Figure 1 – (A) Venn diagram describing the relationship of molecular changes induced by TAM, ICI, TNF α and CAS. Each oval represents the genes that are under the control of the process labeled within the oval. The area where two ovals overlap represents genes that are controlled by both processes. The genes that we are interested in using comparative functional genomics are those represented by black area in the diagram (B) Illustration of the methods used. Multiple copies of the macroarray were made from purified PCR insert products from plasmid clones. Cells were grown and treated as indicated for 48 hours. RNA was extracted from these cultures, double stranded cDNA was synthesized and radioactively labeled by random prime labeling. Different copies of the array were screened with a different labeled cDNA population from the cultures. Shown in the figure is a representative screening of MCF7 cells treated and untreated with TAM.

A



B

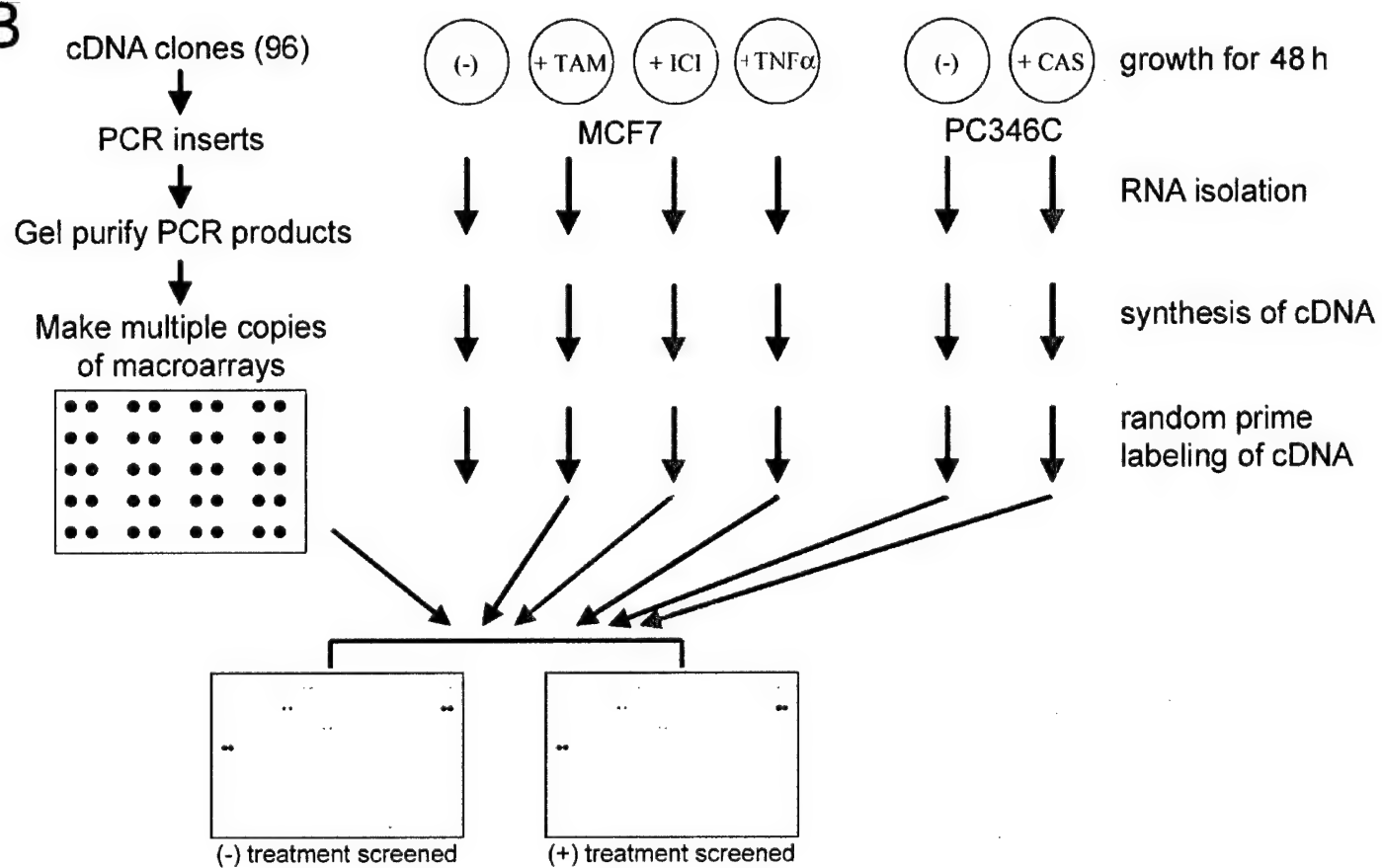


Figure 2 – Treatment of cells with TAM, ICI, TNF α and Casodex lead to a decrease in cell numbers. (A) Cell densities of control MCF7 cells (untreated) or MCF cells treated with TAM (5 μ M), for 24, 48, 72, 96 hours. There is a significant effect on cell numbers as early as after a 48 h treatment. (B) Normalized comparison of cell densities after 96 hour treatments with and without hormone receptor blockers. MCF7 cells and PC346C (prostate cancer) cells were treated with and without hormone receptor blockers (MCF7 cells with 5 μ M TAM and 10 μ M ICI, PC346C cells with 50 μ M Casodex). White bars are indicative of control cell densities which were set at 100% (untreated) Blue bars are indicative of cell densities normalized to control levels after treatment with hormone receptor blockers for 96 hours.

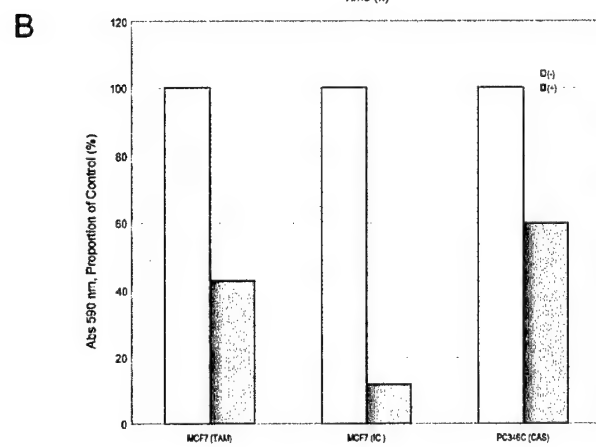
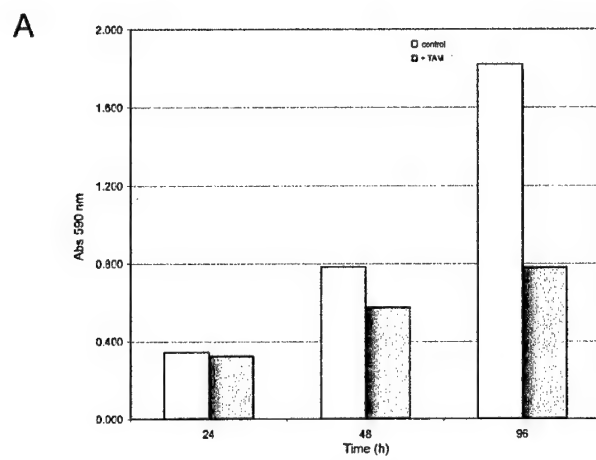


Figure 3 – Reduction in the number of MCF7 cells within 48 hours of treatment with TAM is due to apoptosis. (A) cell sorting results for untreated cells that have been TUNEL labeled. (B) tunnel labeled cells that have been treated with TAM and sorted using FACS analysis. 19.93 % of the cells showed TUNEL labeling over the control.

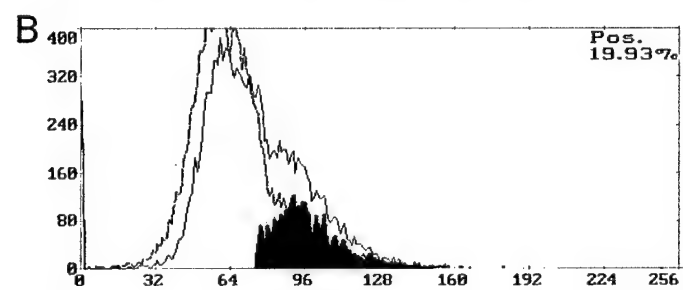
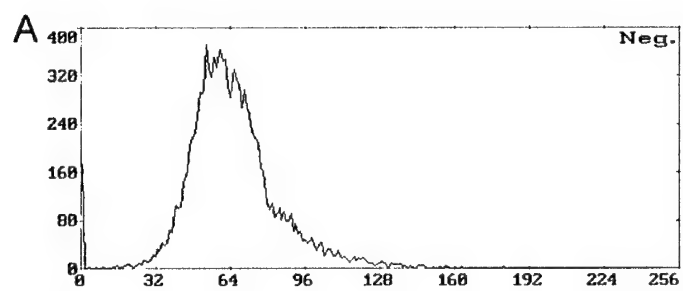


Figure 4 – Expression profiles with TAM and ICI treatment of MCF7 cells and CAS treatment of PC346C cells. Shown are the expression profiles for the 15 clones that were detected with our screens. Blue bars represent the normalized hybridization intensities detected by probes derived from cell cultures treated with the chemical in question for 48 hours. White bars represent the normalized hybridization intensities by the probe derived from the corresponding untreated controls (48 hours). Below each chart the cell type and treatments are indicated as well as a figure of the hybridization spots from the autoradiographs of the screened arrays. Genes that show similar expression profiles are grouped together as indicated by a letter designation and outlined by a solid line.

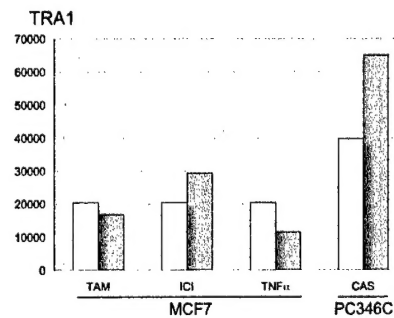
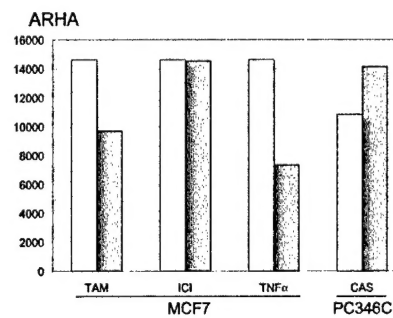
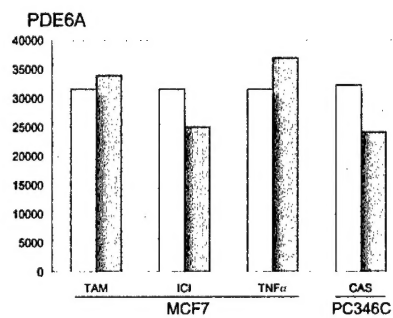
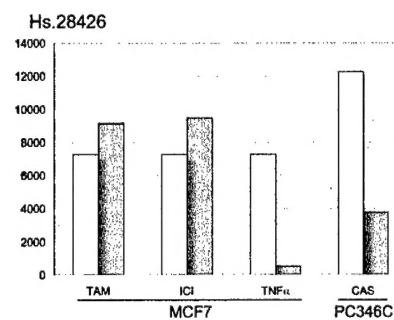
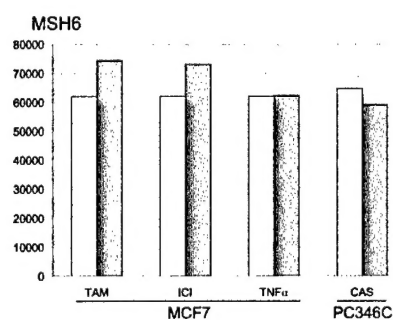
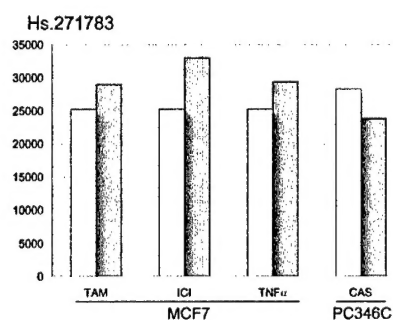
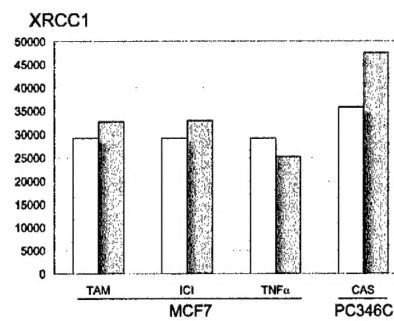
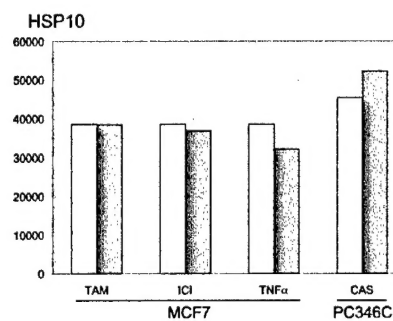
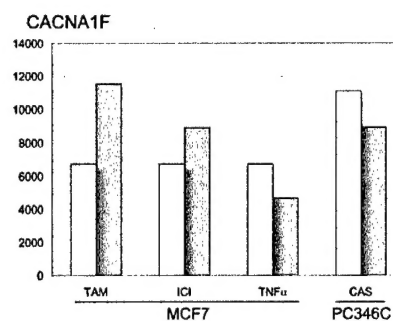
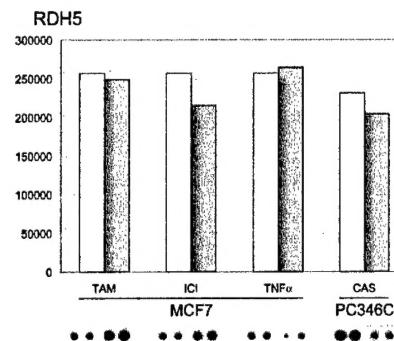
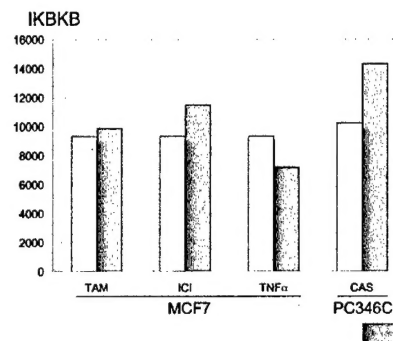
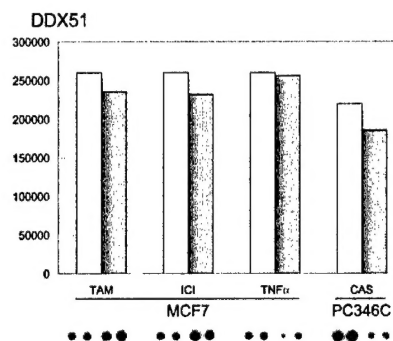
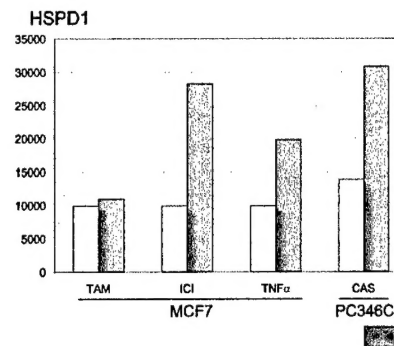
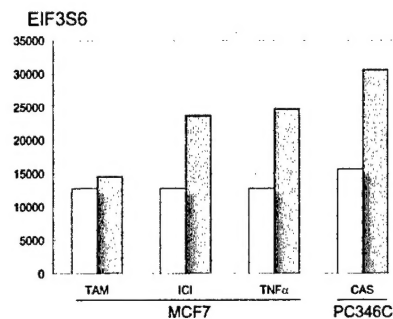
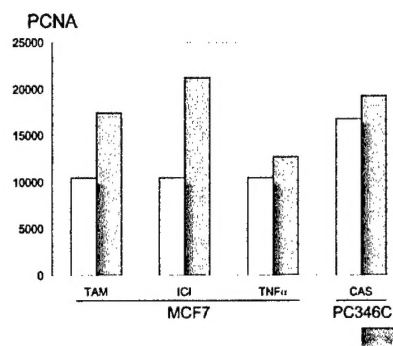
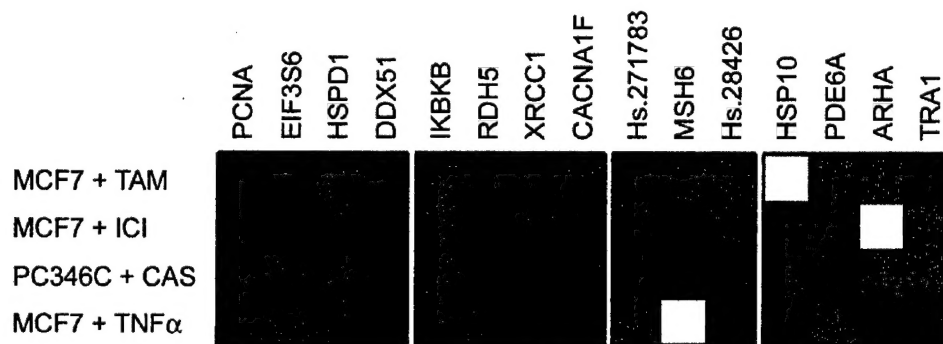


Figure 5 – Analysis of absolute changes in gene expression seen with the 4 treatments that induce apoptosis sensitivity. Green indicates an up-regulation with treatment, red indicates a down-regulation and yellow indicates no change in expression. In total 4 genes were found to have a consistent change in all 4 ACD inducible model systems (PCNA, EIF3S6, HSPD1 and DDX51). Three of these genes (EIF3S6, HSPD1 and DDX51) were not detected using a n-fold analysis.



Gene	MCF7 (-)	MCF7 +TAM	n-fold
RDH5	256876	248943	0.97
CACNA1F	6720	11530	1.72
EIF3S6	12700	14572	1.15
PCNA	10425	17420	1.67
MSH6	61913	74325	1.20
XRCC1	29104	32628	1.12
HSPD1	9896	10950	1.11
HSP10	38524	38462	1.00
TRA1	20373	16756	0.82
IKBKB	9313	9871	1.06
PDE6A	31539	33927	1.08
ARHA	14605	9705	0.66
Hs.271783	25189	28978	1.15
DDX51	259902	235394	0.91
Hs.28426	7250	9125	1.26

Table 1 - Normalized intensities of measurable spots from the macroarrays screened for MCF7 TAM induced differential gene expression. Names of the genes represented by the PCR product spotted on the array are indicated on the left column of the table. In the right column are the n-fold values calculated for treated/control. Those n-fold values equal to or greater than 1.50 or 1/1.50 are bolded.